DIRECT BENEFICIAL EFFECTS OF CYTOKININ-PRODUCING RHIZOBACTERIA ON PLANT GROWTH

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ABSTRACT

A study was conducted to determine the role of cytokinin production by certain plant growth promoting rhizobacteria (PGPR) strains in the regulation and/or promotion of plant growth and development. PGPR were screened for cytokinin production in pure culture and in association with *Raphanus sativus* (radish) cv. Cherry Belle plants and tobacco callus tissues of *Nicotiana tabacum*, var. Xanthi. The cytokinins isopentenyl adenosine (IPA), zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) were measured by immunoassay. The ability of selected PGPR strains to promote plant growth and development was studied in *Triticum aestivum* (wheat) cv. Katepwa and radish grown in growth pouches and in pots in controlled environment chambers.

In initial studies, pure cultures of five PGPR strains and 16 mutants were shown to vary in their growth and production of cytokinins. After 336 h in MM+Gl medium, *Pseudomonas fluorescens* strain G20-18WT, G20-18 rifampicin resistant mutant (RIF) and two Tn5 insertion transconjugants (CNT1, CNT2) produced 8.60, 6.80, 0.90 and 0.80 pmol mL^{-1} of [IPA+ZR+DHZR], respectively. Addition of 10^{-7} M adenine increased cytokinin production in 96 h cultures of strain G20-18WT by 68 %. Although differences were observed among strains, the proportion of IPA produced to the total [IPA+ZR+DHZR] was on average higher than 92% while the percentages of ZR and DHZR were 3.3 and 4.7%, respectively.

Strain G20-18WT and several mutants colonized Katepwa wheat plants similarly (Log 4.2 cfu g^{-1} dry weight) after 21 d. However, only G20-18WT

significantly increased emergence, shoot and root biomass by 14, 22 and 30%, respectively. At anthesis, leaf area, root biomass, number of tillers and visible ears were significantly increased by this strain, however grain yield was not affected.

Radish roots were colonized by all tested PGPR strains, however, only strain G20-18WT increased emergence, root and shoot length and biomass of plants grown in both growth pouches (GP) and greenhouse. Similar effects were observed with exogenous applications of zeatin (Z) ranging between 0.5 and 10 n*M* and cytokinin combinations lower than 0.5 n*M*. Inoculation with G20-18WT increased the concentration of cytokinins in the rhizosphere fivefold. The concentrations of IPA and DHZR in radish rhizospheres and plant tissues inoculated with G20-18WT were twofold higher than in those inoculated with the mutants.

Fresh weight increases (FWI) of tobacco callus cultured in the presence of strains G20-18WT, RIF, CNT1 and CNT2 were 4.8, 4.0, 2.4 and 1.9 times higher than the FWI of callus grown on control plates with a cytokinin-free medium. These increases were correlated with direct and indirect estimates of [IPA+ZR+DHZR] in the medium. The presence of plant callus affected the amounts and proportions of cytokinins produced by the PGPR strains.

These results are the first evidence showing a direct link between plant growth promotion and cytokinin production by a *Pseudomonas* PGPR strain.

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DEDICATION

I want to dedicate this work to my parents, BLAS and AMELIA who instilled in me the sense of perseverance and always were close to me wherever I was.

I especially want to dedicate this thesis and express my gratitude to my husband DANIEL for his always-stimulating attitude, his help to solve the every-day problems with a smile and for his love that is one of the most important things that give me happiness.

I also want to dedicate this thesis to my daughter PAULA because she always teaches me something new and keeps me busy with interesting things.

I hope that they always remember how much I love them.

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LIST OF ABBREVIATIONS

0+0	MS24-2 medium without both 2,4-D and cytokinins
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
Ade	adenine
AMP	adenosine monophosphate
AWCD	average well color development
B plates	bacterial control plates
BAP	6-benzylaminopurine
cfu	colony forming units
CNT	cytokinin negative transconjugants
d	day/s
d.a.p.	days after planting
DHZ	dihydrozeatin
DHZOG	dihydrozeatin-O-glucoside
DHZR	dihydrozeatin riboside
DHZROG	dihydrozeatin riboside-O-glucoside
ELISA	enzyme linked immunosorbent assays
FWI	fresh weight increase
GA_3	gibberellic acid
GC	gas chromatography
GLC	gas liquid chromatography
GP	growth pouches

h	hour
HPLC	high performance liquid chromatography
IAA	indole acetic acid
Ipa	isopentenyl adenine
IPA	isopentenyl adenosine
IPNT	isopentenyl nucleotide
IPP	isopentenyl phosphate
ipt	isopentenyltransferase
Log	logarithm
LSD	least significant difference
M	molar
min	minute/s
mg	milligram
mL	milliliter
mm	millimeter
m*M*	millimolar
MM+Gl	minimal medium with 10 g L^{-1} of glucose
MRM	multiple reaction monitoring
MS	mass spectrometry
N	normal
n.d.	not detectable
nL	nanoliter
n*M*	nanomolar

n.s.	not significant
nmol	nanomole
PBI-NRC	Plant Biotechnology Institute-National Research Council
PC	principal component
PCA	principal component analysis
PGPR	plant growth promoting rhizobacteria
PGR	plant growth regulator
RIA	radioimmunoassay
rpm	revolutions per minute
RRSA	relative root surface area
s	second
SC	side chain
SD+0	MS24-2 medium with 2,4-D but without cytokinins
SD-BAP	standard MS24-2 medium with both BAP and 2,4-D
T plates	tobacco callus control plates
T+B plates	plates with tobacco callus and bacteria grown together
TCB	tobacco callus bioassay
TLC	thin liquid chromatography
Tn	transconjugant
µg	microgram
µL	microliter
µm	micrometer
µmol	micromole

µM	micromolar
VAM	vesicular-arbuscular mycorrhizae
v/v	volume per volume
w/v	weight per volume
Z	trans-zeatin
Z7G	zeatin-7-glucoside
ZE	zeatin equivalents
ZE-cfu	zeatin equivalents expressed as nmol per 10^{10} cfu.
ZNT	zeatin nucleotide
ZOG	zeatin-O-glucoside
ZR	zeatin riboside
ZRNT	zeatin riboside nucleotide

CHAPTER 1. INTRODUCTION

Plant growth regulators (PGRs) are organic substances that influence physiological and developmental processes of plants at very low concentrations (picomoles and/or nanomoles) (Salisbury and Ross 1992). PGRs do not act alone and the final conditions of plant growth and development represent the net effect of a determinate balance of PGRs.

In spite of the fact that plants are capable of synthesizing PGRs, they may also respond to exogenous applications of PGRs during certain phases of growth and under certain cultivation conditions. This is related to the idea that plant growth and development are governed by both PGR concentration and tissue sensitivity (Karmoker and Van Steveninck 1979; Venis 1987).

Cytokinins are endogenous PGRs. N^6-substituted aminopurines are cytokinins present in plants as free base forms or t-RNA constituents. The cytokinin trans-zeatin (Z) and its derivatives are the most ubiquitous cytokinins in plants (Letham 1994). Initially cytokinins were first shown to induce cell division (Miller et al. 1956), however, they are now known to act in combination with other PGRs to regulate diverse responses in plants. Processes such as cell division, accumulation of chlorophyll, leaf expansion and delay of senescence are among processes regulated by cytokinins. Endogenous cytokinins are synthesized in roots and translocated to the shoots in the xylem (Letham 1994).

There is evidence that interactions among PGRs in different environmental conditions can play a role in root-to-shoot communication (Patrick 1987; Rubery 1987). Because much of the chemical information moving from the root to the shoot can be modified as a result of altered root functioning as soil conditions change, it is important to consider that many PGRs or their derivatives are produced by soil microorganisms, specifically by those living in the rhizosphere (Arshad and Frankenberger 1993).

Soil microorganisms that aggressively colonize roots and establish a permanent relationship with roots and promote plant growth represent a subset of rhizosphere bacteria usually called plant growth promoting rhizobacteria or PGPR (Kloepper 1993). PGPR should survive on seeds or soil after inoculation, multiply in the rhizosphere in response to seed exudates and attach to the root surface to colonize the developing root system (Bolton et al. 1993). PGPR can produce direct or indirect effects on host plants. Indirect effects are those related to the production of substances, such as antibiotics, siderophores or HCN. These substances increase growth by decreasing the activities of pathogens or deleterious microorganisms. On the other hand, PGPR can produce direct effects on plant growth when they produce metabolites, such as PGRs, that directly promote plant growth and/or development without interactions with native soil microflora (Kloepper 1993). PGR production by PGPR has been demonstrated in culture media and soil (Arshad and Frankenberger 1993). Strains of *Azospirillum* (Morgenstern and Okon 1987; Fallik et al. 1989, García de Salamone et al. 1996), *Azotobacter* (Nieto and Frankerberger 1989),

Pseudomonas (Glick et al. 1994; Young et al. 1990), *Serratia* (Zhang et al. 1997) and *Bacillus* (Kucey, 1988; Muller et al. 1988) have been identified as PGPR producing direct effects on plant growth and/or development.

A core collection of elite *Pseudomonas* PGPR strains was screened for PGR production and a positive correlation between root elongation and cytokinin production was observed for certain strains (Young et al. 1990). The ecology of *Pseudomonas* PGPR is a relatively new research area and, although plant growth can be modified by these PGPR, further research is required with respect to their mode of action in order to explain specific inoculation responses. Extensive investigations were conducted on cytokinin synthesis in pure cultures of PGPR. However, little work has been done to elucidate the effects on plant growth of cytokinins exogenously produced by soil microorganisms. The rhizosphere is densely populated with microorganisms because it is rich in carbon and nutrients as a result of rhizodeposition (Paul and Clark 1989; Lynch 1990). Microbially-derived PGRs, such as cytokinins, could regulate plant growth and provide an inexpensive and continuous source of PGRs for plant uptake.

Because cytokinins are involved in several growth and developmental processes, the hypothesis formulated for this project is: **cytokinins derived from certain *Pseudomonas* PGPR can regulate and/or promote plant growth and development.**

Three general objectives have been enunciated in order to study the formulated hypothesis:

1. Identify a plant system that is highly sensitive to exogenous applications of cytokinins.
2. Determine the relationship between cytokinin production by PGPR strains and effect on plant growth.
3. Estimate the amount and type of cytokinins produced by PGPR strains in the presence of plant tissue.

CHAPTER 2. LITERATURE REVIEW

2.1. Plant Growth Regulators.

Plant growth regulators (PGRs) are organic substances that influence physiological and developmental processes of plants at very low concentrations. They are often effective at internal concentrations lower than 1 μM, whereas amino acids, organic acids, sugars, and other metabolites necessary for growth and development are usually present at concentrations of 1 to 50 m*M*.

Auxins, gibberellins, cytokinins, ethylene and abscisic acid (ABA) are the five major groups of PGRs, usually called phytohormones, synthesized endogenously by plants and their effects on plant growth and development have been reviewed elsewhere (Salisbury and Ross 1992; Arshad and Frankenberger 1993). Brassinosteroids and polyamines are also PGRs endogenously synthesized by plant tissues and have been reviewed recently by Sasse (1991) and Galston and Sawhney (1990), respectively. PGRs also include synthetic compounds that cause many physiological responses when they are exogenously applied to plant tissues (Salisbury and Ross 1992).

It is known that PGRs do not act alone and the final condition of plant growth or development represents the net effect of a determinate balance of endogenous PGRs. However, PGR effects have been elucidated largely from exogenous applications. In spite of the fact that plants are capable of synthesizing

PGRs, they may also respond to exogenous applications during certain growth phases and under certain cultivation conditions. Plant growth and development are likely to be governed both by PGR concentration and by tissue sensitivity to the PGR (Venis 1987). Based on the analyses of Trewavas (1987) and Simpson (1990), PGRs can be studied as exogenous or endogenous chemical factors that act on physiological systems to facilitate synchronism of their development.

2.2 Cytokinins

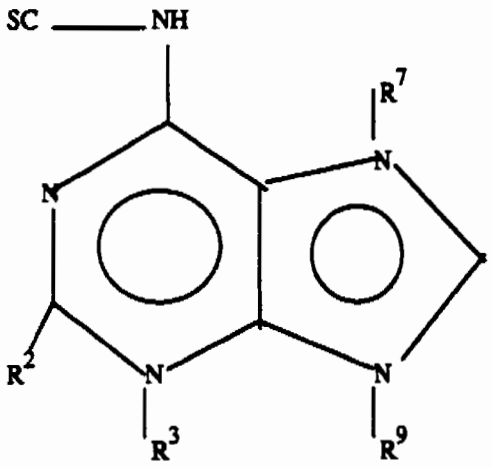
2.2.1. Structure, Metabolism and Compartmentalization

Cytokinins are PGRs that regulate cytokinesis in plant cells (Skoog et al. 1965). Following the discovery of kinetin (6-furfurylaminopurine), a compound isolated from autoclaved herring sperm DNA that had potent cell-division-promoting activity (Miller et al. 1956); large numbers of analogous N^6-alkylaminopurines were synthesized. Studies concerned with the relationship between chemical structure and activity have been reviewed by Shaw (1994). When a naturally occurring kinetin-like compound was isolated from maize and sunflower fruits and soybean (Miller 1961) its properties suggested that it was a purine derivative. However, the first naturally occurring pure crystalline cytokinin was isolated from *Zea mays* and given the name zeatin (Z) by Letham (1963). Several tentative structures were proposed (Letham et al. 1964) but the correct one, namely (E)-4-(hydroxy-3-methyl-but-2-enyl)aminopurine was confirmed by chemical synthesis (Shaw and Wilson 1964) (Fig. 2.1). The natural occurrence of Z was soon confirmed both in *Z. mays* and in many other sources (Letham

1978). Following this early work, several new cytokinins were isolated from natural sources and most of them were N^6-substituted adenine or substituted derivates and N- or O- glycosides and their phosphorylated derivatives (Letham 1978; Letham 1994; Shaw 1994) (Fig. 2.1).

In addition to their widespread occurrence in plant tissues, numerous cytokinins have also been isolated from t-RNAs of virtually all organisms (Skoog and Armstrong 1970). Possible functions of modified nucleosides of tRNA include influencing tRNA structure, providing recognition sites, affecting the efficiency and accuracy of translation, and having a regulatory role. Only modifications found in position 37 are the hypermodified, hydrophobic isopentenyl adenosine (IPA) derivatives (Fig. 2.1) known as cytokinins. (Taller 1994), and their distribution among organisms seems to show inter-kingdom differences (Skoog and Armstrong 1970; Greene 1980; Sprinzl et al. 1991).

Nowadays, it is accepted that cytokinins can be structurally classified into two categories: the adenine cytokinins (Fig. 2.1) and the diphenylurea cytokinins (Fig. 2.2) (Shaw 1994; Shudo 1994). N-phenyl-N'-(4-pyridyl)urea, N-(2-chloro-4-pyridyl)-N'-phenylurea and N-phenyl-N'-(1,2,3-thiadiazol-5-yl)urea exhibit essentially the same activities as 6-benzylaminopurine (BAP) or Z in several bioassays and intact plants (Okamoto et al. 1978; Iwamura et al. 1979; Mok et al. 1987b; Reynolds 1987; Karanov et al. 1992). Both types of cytokinins have similar structure-relationships, and similar biological activity suggesting that



SC1: -CH2-CH=CH-CH3
|
CH3

SC2: -CH2-CH=C-CH2-OH
|
CH3

SC3: -CH2-CH2-CH-CH2-OH
|
CH3

Adenine (Ade) Structure

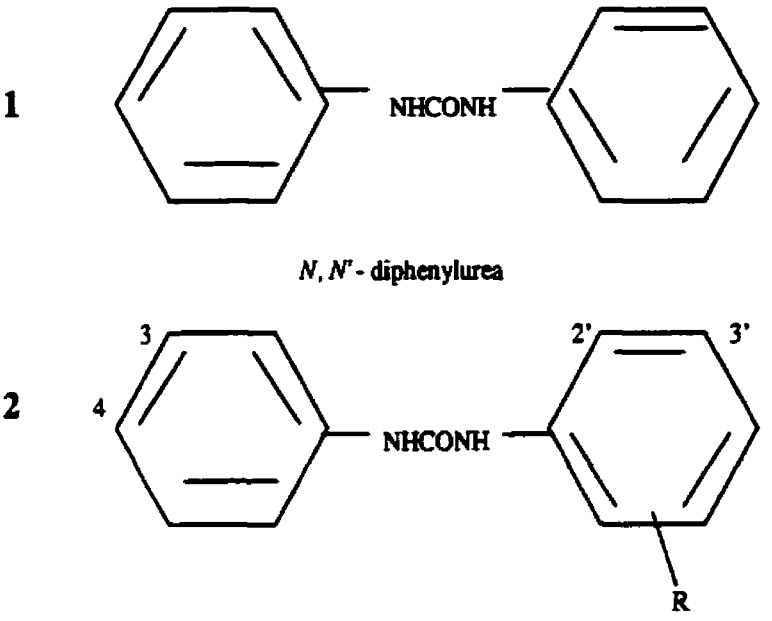
Modifications of the Ade Structure

Form	Abreviation	Type of Side Chain (SC)	Moiety attached at indicated N position
Base	IPa	SC1	None
	Z	SC2	None
Riboside	IPA	SC1	Ribose at R^9
	ZR	SC2	Ribose at R^9
Nucleotide	IPNT	SC1	Nucleotide at R^9
	ZRNT	SC2	Nucelotide at R^9
N-Glucose (G) conjugate		SC1 / SC2	G attached at R^3, R^7 R^9 .
N-Alanine (A) conjugate		SC1 / SC2	A at R^9

Modifications of Side Chain

Form	Abreviation	Type of SC	Moiety attached at indicated N position
Dihydro-derivatives	DHZ	SC3	None
	DHZR	SC3	Ribose at R^9
O-Glucosyl derivatives	ZOG	SC2-G	None
	DHZOG	SC3-G	None
O-Acetyl (Ac) derivatives	OAcZR	SC2-Ac	Ribose at R^9
	OAcDHZ	SC3-Ac	None
O-Xylosyl (X) derivatives	OXZ	SC2-X	None
	OXDHZ	SC3-X	None

Figure 2.1. Adenine cytokinins and derivatives after modifications of the purine structure and side chain. Compiled from Jameson (1994); Kaminek (1992), Brzobohaty et al. (1994).



2a: *N*-3-fluorophenyl-*N'*-4-fluorophenylurea
2b: *N*-4-nitrophenyl-*N'*-4-phenylurea
2c: *N*-4-nitro-3-trifluoromethylphenyl-*N'*-phenylurea
2d: *N*-3,4-dichlorophenyl-*N'*-phenylurea
2e: *N*-phenyl-*N'*-4-pyridylureas [1]

Figure 2.2. Basic structures of the diphenylurea cytokinins
Compounds (2a-2e) have consistently high or moderate activity in the initiation of cell division of tobacco pith but weak activity in the standard tobacco callus bioassay (Takahashi et al. 1978). R: indicates possible substitutions of Cl, F, Br, CH_3O, OH or NH_2 on the *N'*-phenylurea ring which greatly enhance the activity. [1]: The pyridyl radical also can be attached to the positions 2, 3 of *N'*-phenylurea ring.

adenine and urea cytokinins are agonists and that both types of cytokinins may act through a common receptor (Shudo 1994).

Elucidation of the metabolic pathways of cytokinins has been largely based on the use of exogenous radiolabeled cytokinins (Letham 1994). Cytokinins exogenously applied to plant tissues usually are rapidly distributed among the respective nucleotide, nucleoside and base forms (Fig. 2.1), but are ultimately broken down either by side chain cleavage to the corresponding adenine derivates which are irreversibly inactived or by conjugation into storage or inactive forms (Fig.2.3) (Jameson 1994). Several comprehensive reviews on cytokinin metabolism have been published elsewhere (Letham and Palni 1983; McGaw et al. 1984; McGaw and Horgan 1985; Kaminek 1992; Brzobohaty et al. 1994; Frankenberger and Arshad 1995).

As the formation of free cytokinins begins with biosynthesis of the isopentenyl nucleotide (IPNT) from isopentenyl phosphate (IPP) and adenosine monophosphate (AMP) (Fig. 2.3), it is known that a considerable amount of interconversion of free bases, ribosides, and nucleotides is possible. BAP appears to be extensively converted to the corresponding ribonucleoside, ribonucleotide and glucoside, in the tissues of several plants (Murai 1994). The enzyme systems catalyzing the interconversions of cytokinin base-riboside-nucleotides appear to be the same as those for Ade-adenosine-AMP interconversions (Chen 1982). However, the distribution and activity of these enzymes change markedly during development, suggesting that these enzymes regulate cytokinin activities (Burch and Stuchbury 1987).

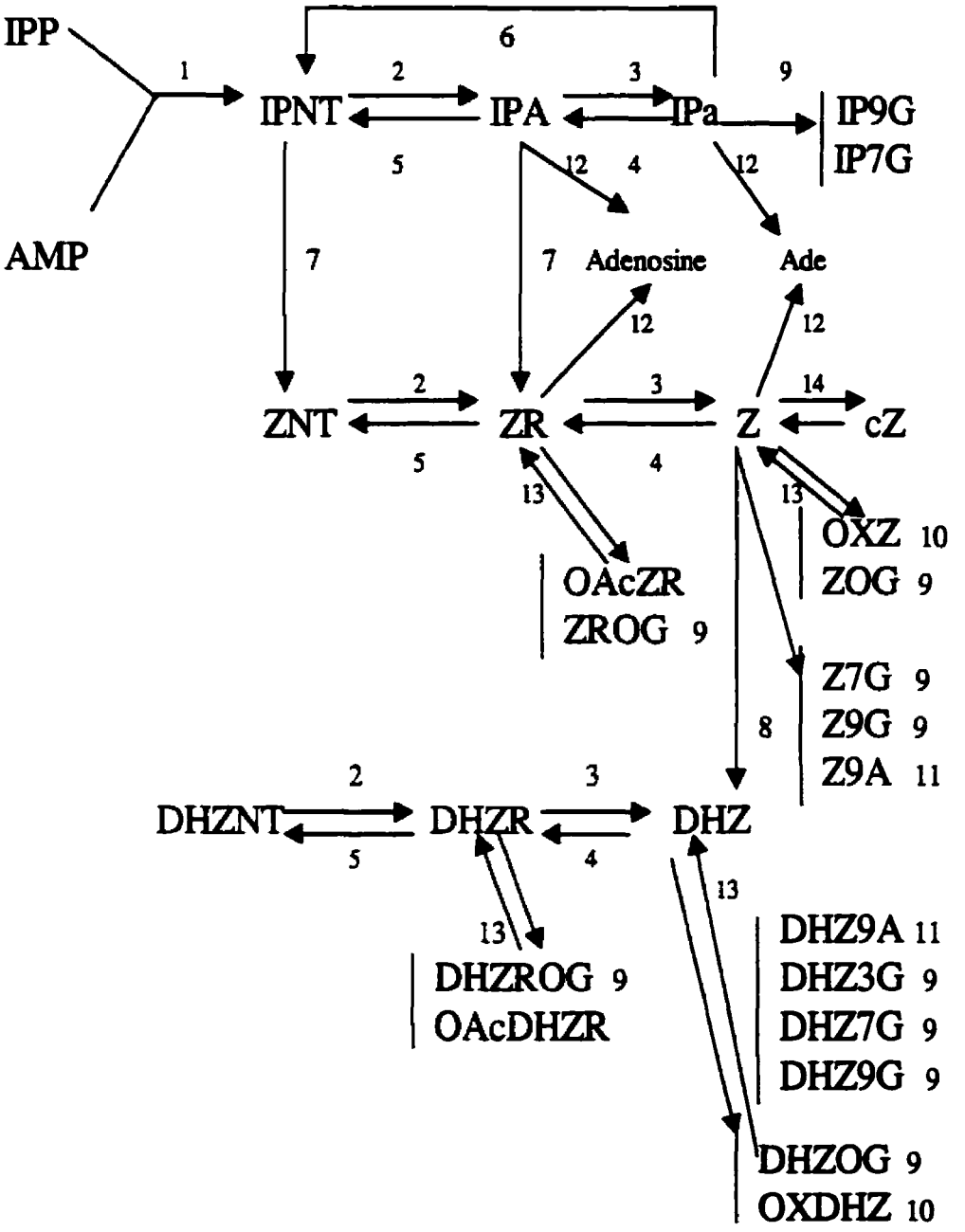


Figure 2.3. General biosynthetic pathways of cytokinins. Number besides the arrows or specific compounds indicate the enzyme involved in the reaction. 1 Isopentenyltransferase, 2 5'nucleotidase/nonspecific phosphatases, 3 Ade nucleotidase, 4 Ade phosphorylase, 5 Ade kinase, 6 Ade phosphoryl transferase, 7 mixed function oxidases, 8 Z reductase, 9 O-glucosyl transferase, 10 O-xylosyl transferase, 11 β-(9-cytokinin alanine)synthase, 12 cytokinin oxidase, 13 β-glucosidase, 14 Z *cis-trans* isomerase. Abreviations of cytokinin compounds as described in Fig. 2.1. Adapted from Jameson (1994) and Brzobohaty et al. (1994).

Glucosylation of cytokinins can occur at the 3, 7, and 9 position of the purine moiety (Letham and Palni 1983) (Figs. 2.1. and 2.3). The 7- and 9- glucosides of Z and BAP appear to be stable metabolites in the tissues in which they are formed and they are relatively inactive in bioassays and resistant to hydrolysis by β-glucosidase (Letham 1994). In general, the 7- and 9- glucosides are unlikely to be storage forms because of their low turnover *in vivo* and their formation does not appear to be coupled to cytokinin activity (Letham and Gollnow 1985). Consequently, these metabolites are generally regarded as detoxification or deactivation products (McGaw et al. 1984). Alanine conjugates at the 9 position of the purine ring (Fig. 2.1) such as lupinic acid and dihydrolupinic acid were unequivocally identified in lupins, and have only been identified in members of the *Fabaceae* as potential storage forms rather than deactivation products (Letham et al. 1990).

In general, the side chain of adenine cytokinins can be modified by reduction or cleavage (Jameson 1994) (Fig. 2.1). It was shown that reduction enhances structural stability as dihydroderivatives are not usually substrates for cytokinin oxidase and conjugation of these compounds provides a mechanism to control their biological activity (McGaw et al. 1985). Oxidative cleavage of the side chain at N^6 to produce adenine, adenosine and adenine nucleotides (Fig. 2.3) is the predominant fate of exogenously supplied Z and zeatin riboside (ZR) and isopentenyl adenine (IPa) (Fig. 2.1) in various plant tissues and results in irreversible destruction of cytokinin activity (Jameson 1994). The cytokinin oxidases are the enzymes involved in this uncharacterized degradation process,

which is an important component in the metabolic network that controls the levels of cytokinin metabolites and their distribution in plant systems (Armstrong 1994). The side chain of adenine cytokinin can also undergo several types of conjugations. O-glucosylation occurs in different plant tissues (Fig. 2.1) (Jameson 1994; Kaminek 1992). The O-glucosides are ubiquitous endogenous compounds present in developing, mature and senescent tissues (Letham and Palni 1983). By feeding O-glucosides to plant tissues McGaw and Horgan (1983) showed that O-glucosides are not substrates for cytokinin oxidase, but are readily degraded by β-glucosidases and unlike N-glucosyl conjugates, are readily metabolized in several plant systems (Jameson 1994; Mok et al., 1987a). The O-glucosides also decline following induction of specific phases of plant development such as fertilization of the wheat ovule (Letham 1994), seed germination (Smith and Van Staden 1978), and bud growth (Van Staden and Dimalla 1978). McGaw et al. (1985) have shown that the O-glucoside moiety can be cleaved when O-glucosides of Z, dihydrozeatin (DHZ) and dihydrozeatin riboside (DHZR) (Figs. 2.1 and 2.3) were fed to de-rooted radish seedlings. These authors noted that Z7G (Fig. 2.1) was susceptible to cytokinin oxidase *in vitro* and they suggested that the apparent metabolic stability of Z7G might be due to compartmentalization because the levels of the oxidase enzymes were high. In many tissues Z and/or ZR accumulate to relatively high levels, which because of their marked susceptibility to cytokinin oxidase, is indicative of some form of compartmentalization.

The discrepancies observed between the nature of endogenous cytokinins and the metabolic fate of exogenous cytokinins might be explained by invoking compartmentalization of either the cytokinins or the metabolic enzymes (Jameson 1994). However, Fusseder and Ziegler (1988) performed the only study where compartmentalization was directly addressed. They observed that the predominant metabolites of ^{3}H-DHZ supplied to photoautotropic suspension cultures of *Chenopodium rubrum* were DHZOG and DHZROG, (Fig. 2.3). These metabolites were compartmentalized within the vacuole, while DHZ and DHZR were localized predominantly outside the vacuole.

Recently, the key properties of enzymes involved in cytokinin metabolism (Mok and Martin 1994; Jameson 1994) and cytokinin degradation, primarily cytokinin oxidases (Armstrong 1994) have been reviewed, revealing that the metabolic picture is extremely complex (Fig. 2.3). There are not only obvious variations among species, but also differences among organs and even tissues of the same plants. In some plants metabolism is similar, irrespective of the supplied cytokinin, whereas in others it varies depending on the substrate (Jameson 1994). In several species, the metabolism of cytokinins changes with age (Van Staden et al. 1988). Extensive studies carried out on several plant species have revealed that metabolic differences exist even at the organ and tissue levels (Turner et al. 1985; Hocart and Letham 1990). Thus, Fig. 2.3 describes a general scheme of metabolic pathways for cytokinins in plants and specific studies of the biosynthesis and metabolism of any particular system should be defined on the basis of this knowledge.

In summary, the level of active cytokinin at a particular site of action may be influenced by a large number of factors including synthesis, oxidative degradation, formation and hydrolysis of inactive conjugates, transport into and out of particular cells and sub-cellular compartmentalization to or away from sites of action.

2.2.2. Cytokinins in Plant Tissues and Their Role in Growth and Developmental Processes

Cytokinins have been found in roots, stems, leaves, flowers, fruits and seeds and are probably present in all living cells of intact higher plants (Salisbury and Ross 1992). The extremely low levels of the endogenous cytokinins in plant tissues and the central role of the most likely precursors in cellular metabolism have made it difficult to determine the sites of cytokinin biosynthesis (Letham 1994). However, strong evidence of different types indicates that the root is the main site of cytokinin biosynthesis (Carmi and Van Staden 1983; Goodwin et al. 1978; Henson and Wareing 1976; Letham 1978; Neuman et al. 1990; Nooden and Letham 1993). Biosynthesis occurs mainly in the root apex and is the result of an interaction between the quiescent center and the adjoining proximal meristem (Feldman 1979). Although no sites of cytokinin biosynthesis have been established unequivocally in the shoot of an intact plant (Letham 1994; Brzobohaty et al. 1994) evidence substantiates the view that cotyledons are supplied with cytokinins from the embryonic axis (Hutton et al. 1982; Nandi et al. 1988).

Recent studies showed a great variation in the principal endogenous cytokinins moving from the root and found in xylem sap of diverse species, and no clear characterization could be done (Letham 1994). Cytokinins move from roots and embryonic axis to other tissues to control diverse aspects of development by interacting with others PGRs. PGR signals moving from root to shoot probably modulate those translocated from shoot to root and vice versa, influencing the appropriate balance of PGRs between root and shoot, which may be responsible for the close correlation between root and shoot growth.

Different types of PGRs can act synergistically or antagonistically; for instance, cytokinins delay leaf senescence, but so do auxins and gibberellins, whereas abscisic acid and ethylene promote senescence (Arshad and Frankenberger 1993; Nooden et al. 1990a). Even though it may be difficult to separate the influence of cytokinins from that of other PGRs or the environment, in this section some examples of interaction between cytokinins and other PGRs are included.

One of the most important experiments involved the growth of tobacco callus on a medium containing different ratios of auxin and cytokinin (Skoog and Miller 1957). These authors demonstrated that plant tissues could be made to differentiate into shoots or roots, by altering the ratio of auxin and cytokinin.

Many factors regulate cell division in plants and it is expected that PGRs such as cytokinins interact with them in the regulation of cell division (Trewavas 1987). Cytokinin and ABA influence total ribosomal protein phosphorylation in plants. This occurs inside the ribosome initiation center, which is thought to play

an important role in the early steps of the initiation of translation (Trewavas 1985). Since the action of BAP and ABA on S_6-ribosomal protein phosphorylation correlates with PGR effects on polysome formation in cotyledon cells, it is possible that S_6 phosphorylation is involved in the mechanism of hormonal regulation of RNA translation in plants (Bergounioux et al. 1988; Ordas et al. 1992).

Although the role of auxins as the main regulatory factors in vascular differentiation is well established, cytokinins, acting only in the presence of the auxin, indole acetic acid (IAA) plus gibberellic acid (GA_3), are both limiting and controlling factors in the early stages of fiber differentiation of explants (Aloni 1987).

Das et al. (1956) reported that the major feature of cytokinin action was the enhancement of cytokinesis in bi-nucleate cells resulting from the presence of auxin alone. This was confirmed later in many other studies using a variety of cultured plant materials (Jacqmard et al. 1994). Evidence suggests that PGRs such as gibberellins (Sachs 1965; Banerjee 1968), ethylene (Apelbaum and Burg 1972; Lieberman 1979) and polyamines (Tabor and Tabor 1984; Evans and Malmberg 1989; Pfosser et al. 1990; Maki et al. 1991) participate in the regulation of the cell cycle in plants. However, there is no evidence of the possible interactions between cytokinins and these PGRs.

Several authors have shown that a number of cytokinin-responsive genes respond to other PGRs, including auxins (Boerjan et al. 1992; Crowell and Amasino 1991), gibberellic acid (Lips and Roth-Berejano 1969), abscisic acid

(Lu et al. 1992), and ethylene (Simmons et al. 1992). These observations are consistent with the concept of synchronism of development (Simpson 1990) and with the hypothesis that relative concentrations of many PGRs, rather than absolute levels of individual PGRs, determine developmental processes (Salisbury and Ross 1992). Numerous cytokinin-inducible or cytokinin-repressible gene products accumulate in response to stress or are related to gene products that respond to stress and are strongly expressed in roots (Crowell and Amasino 1991; Simmons et al. 1992; Shinshi et al. 1987). Although cytokinins influence molecular processes that are known to participate in signal transduction, the role of these processes in cytokinin action is still unclear and the mechanism by which cytokinins regulate the expression of plant genes individually or in interaction with other PGRs remains largely unexplored (Crowell and Amasino 1994).

2.2.2.1. Plant responses to exogenous applications

Cytokinins play a major or minor role throughout development, from seed germination to leaf and plant senescence and modulate physiological processes important throughout the life of the plant, including photosynthesis and respiration (Salisbury and Ross 1992; Mok 1994; Arshad and Frankenberger 1993).

Most of what is known today about the role of cytokinin in growth and development was determined by exogenous application experiments (Klee 1994). Considerable evidence has accumulated which is summarized below.

Exogenously supplied cytokinins stimulate cell division and other treatments that lead to mitotic activity invariably increase the level of endogenous cytokinin (Jacqmard et al. 1994).

Early after the discovery of kinetin, many reports linked cytokinin responses by tissues to the inhibition of respiration and prevention of senescence (Richmond and Lang 1957; Mothes and Engelbrecht 1961). These early observations have been confirmed by numerous studies involving tissues and whole plants of diverse species and an array of cytokinins (Thimann 1980; Van Staden et al. 1988; Singh et al. 1992). Also, conclusive evidence that endogenous cytokinins are involved in control of sequential leaf senescence of tobacco was obtained using exogenous applications of cytokinins (Letham et al. 1990; Nandi et al. 1988). The broad range of oxidative processes controlled by cytokinins has been divided into five main effects: senescence inhibition, cell growth, secondary-compound metabolism, respiration inhibition during senescence and stimulation of respiration during development (Musgrave 1994).

Treatment with kinetin resulted in the movement of nutrients from the untreated to the treated part of the tobacco leaf (Leopold and Kawase 1964) and application of cytokinins raised the osmotic concentration in radish (Longo et al. 1978) and watermelon (Norris 1976) cotyledons. More recent studies indicate that the change in nutrient allocation may be an indirect result of the increase in cytokinin concentration while the increase in wall cell extensibility may be the primary effect (Nielsen and Ulvskov 1992).

The variability of cytokinin effects suggests that these PGRs might have different mechanisms of action in different tissues, or that they have a common primary effect, which is followed by numerous secondary effects that depend on the physiological state of the target cells (Salisbury and Ross 1992). As with other PGRs, amplification of the initial effect must occur because cytokinins are present in such low concentrations ranging between 0.01 and 1 μM. Plant cells contain all the elements for a calcium-based signal transduction chain that can couple the stimulus of cytokinin to its physiological responses, but a unified model is lacking (Saunders 1994). In addition, the nature of cytokinin-binding proteins and receptors remains confusing and controversial (Brinegar 1994).

The level of active cytokinin at a particular site of action is influenced by metabolism and there is also the possibility that physiological responses may be modulated by variations in the ability of cells to respond to a particular concentration of free cytokinin (Trewavas 1992). It is widely accepted that the variability of the cytokinin effects is due to the fact that the exogenous PGR application experiments are subject to many complications that make interpretation of the results difficult (Letham 1994).

A better approach came with the quantification of endogenous cytokinins in the stem and then the introduction of these natural compounds in radioactive forms, in concentrations similar to those occurring endogenously. The next section emphasizes and summarizes those studies.

2.2.2.2. Involvement of endogenous compounds

The level of endogenous cytokinins is generally low in dry seeds and initially decreases but then increases during germination (Thomas 1992). Cytokinins are synthesized in the embryo axes of germinating seeds and transported to the cotyledons of legume crops (Nandi et al. 1988; Van Onckelen et al. 1977; Revilla et al. 1988), squash (Penner and Ashton 1967) and black gram (Morohashi 1982) seeds. The embryo axis is important in mobilizing storage proteins in the cotyledons and cytokinins could replace the axis (Ilan and Gepstein 1981; Pino et al. 1991).

Outgrowth of lateral buds is generally prevented by a correlative signal from the apical bud, whose removal eliminates this signal and allows the growth of laterals shoots (Salisbury and Ross 1992). The endogenous levels of cytokinins have been related to branching patterns showing that the non-branching tomato mutants contained lower levels of cytokinins than the normal branching wild type (Mapelli and Lombardi 1982; Sossountzov et al. 1989). Transformants of tobacco and petunia, containing high cytokinin levels due to the overexpression of the isopentenyltransferase (*ipt*) gene were more branched than the non-transformed control (Klee 1994). Further evidence implicating cytokinins in the release of apical dominance have been derived from studies of cytokinin concentrations in different types of buds such as terminal buds of tomato (Sossountzov et al. 1989) and a water fern (Pilate et al. 1989). These contained more Z and ZR than the quiescent lateral buds and the levels of these cytokinins decreased in lateral buds, proportionally to their distance from the apical bud.

Kuraishi and Okumura (1956) were the first to show that cytokinins have the ability to stimulate leaf expansion. This was subsequently confirmed in a number of other studies (Leopold and Kawase 1964; Brock and Cleland 1990; Letham 1994). Analyses of leaf cytokinins established a correlation between endogenous cytokinins and leaf expansion (Ulstov et al. 1992). Although the mode of cytokinin action in flowering is unclear, it is known that cytokinins influence development of the reproductive organs, stamen and pistil in monoecious and dioecious species (Durand and Durand 1994). The cytokinin contents in apices of isogenic lines of *Mercurialis annua* showed the most dramatic difference between male and female lines due to the presence of Z nucleotide (ZNT) in male apices and Z in female apices (Dauphin-Guerin et al. 1980; Louis et al. 1990; Letham and Palni 1983).

Endogenous cytokinins from a variety of cereal tissues and intact plants have been identified and changes in cytokinin content have been correlated with developmental or growth processes (Jameson et al. 1982; Saavedra-Soto et al. 1988; Ambler et al. 1992). The regulation of these processes in cereals is of particular interest because of their importance in human and animal nutrition (Banowetz 1996).

Cytokinin stimulation of cell division is a direct and non-inductive effect; this means that it is not only required to initiate cell proliferation in non-dividing tissues, but also its continued presence is needed to sustain mitotic activity (Fosket et al. 1977; Meyer and Cooke 1979; Wang et al. 1981). The general

conclusion emerging from all these studies is that cytokinins are one of the major controlling factors of the cell cycle in cultured plant materials.

Cytokinins may act at more than one step of the cell cycle through shortening of the duration of one or more phases of the cell cycle (Houssa et al. 1990; MacLeod 1968). Certain tissues in plants contain suboptimal levels of cytokinin and the progress of their cells through the cell cycle is either slowed or arrested (Nishinari and Syono 1986).

The ability to create transgenic plants was a significant advance in plant biology because this technology has revolutionized approaches in plant physiology and biochemistry (Klee 1994) and has provided convincing evidence linking endogenous cytokinins to plant development (Mok 1994). Transgenic plants offer an alternative that allows for reproducible PGR perturbations, which in theory can be manipulated both spatially and temporally. Up to now, the study of cytokinin biology using transgenic plants has been limited to the use of a gene encoding the enzyme isopentenyl transferase *(ipt)* that synthesizes IPNT (Fig. 2.3). The expression of the *ipt* gene in transgenic plants causes cytokinin overproduction and the plants exhibit various degrees of abnormal morphology because of their altered metabolic systems (Brzobohaty et al. 1994).

Genetic analysis of plant mutants, over the last 20 years, has resulted in a better understanding of PGR biosynthesis and their mode of action (King 1988; Klee and Estelle 1991; Reid 1993). In contrast to other PGRs, cytokinin mutants have been limited to *Nicotiana* and *Arabidopsis*. This may be due to the lack of spontaneous mutants and knowledge about cytokinins, and the fact that the

identification of screening targets is the most difficult process of isolating cytokinin mutants (Wang 1994).

Another alternative to altering plant metabolism is to consider cytokinin production by soil microorganisms that can establish close associations with plant roots. This topic is reviewed in section 2.4.2.

2.2.3. Analysis of Cytokinins

Soon after the identification of kinetin and its effects on cell division and callus growth, there followed a flurry of research effort in the early 1960s to develop cytokinin bioassays (Shaw 1994). Bioassays have been indispensable for the detection of cytokinin activity; in fact, the discovery of cytokinins depended on the tobacco pith bioassay devised by Murashige and Skoog (1962). This bioassay served to evaluate the activities of numerous compounds (Salisbury and Ross 1992). Linsmaier and Skoog (1965) developed an optimized culture medium for the culture of all types of plant tissues including gymnosperms, monocots and dicots (Mok 1994). Other callus bioassays also have been utilized, such as the soybean (Miller 1968), carrot (Shaw et al. 1971), and *Phaseolus* (Mok et al. 1980, 1987b) bioassays. Their use has facilitated the delineation of structure-activity relationships such as the optimal length of the N^6-side chain and the effects of ring-structure modifications (Skoog and Armstrong 1970) (Fig. 2.1).

Although no bioassay was designed for whole plant systems, a considerable number of bioassays have been devised based on the various biological effects of

cytokinins. They include, but are not limited to the following bioassays: the lettuce seed germination assay was developed based on the relationship between kinetin and red-light promotion, which was first reported by Miller (1958). The *Funaria protonemata* (Hahn and Bopp 1968) and pea lateral bud (Thimann and Sachs 1966) bioassays are related to the ability of cytokinins to promote formation of new buds and release buds from apical dominance, respectively. The etiolated bean leaf disc (Miller 1963), *Spirodela* frond expansion (Letham 1967) and radish cotyledon (Letham 1971) bioassays were based on the activity of cytokinins on leaf and cotyledon expansion. Senescence bioassays were devised with various plant species (Letham 1967; Osborne and McCalla 1961; Letham et al. 1983). The *Amaranthus* bioassay measures the formation of betacyanin (Biddington and Thomas 1973; Kohler et al. 1987); and the cucumber cotyledon bioassay (Fletcher et al. 1982) depends on the formation of chlorophyll.

Specificity, high sensitivity and detection of minute quantities are the essential attributes that determine the efficacy of a bioassay (Skoog and Armstrong 1970). In addition, the bioassay should be quantitative to allow comparisons to the activity of known standards (Letham et al. 1983). Callus bioassays are generally specific, sensitive and quantitative but require a long assay time. A comparison of the activities of Z-derived cytokinins in four different cytokinin bioassays showed that the tobacco callus bioassays had the highest sensitivity (Letham et al. 1983).

Bioassays have been used to assess cytokinin activity of new compounds as well as to determine the cytokinin activity of plant extracts. For the former use, they were, are and will be indispensable, since they offer the only means of determining whether a compound, either directly or indirectly, exerts cytokinin activity. For the latter use, bioassays were essential in the earlier period of cytokinin research, but in recent years they have been replaced by more precise methods of cytokinin analyses, such as chromatography followed by immunoassays or quantitative mass spectra analyses.

During the first two decades of cytokinin research, numerous analytical procedures were used for the detection, isolation and identification of cytokinins, but few of them have been developed specifically for cytokinins and in general they have been borrowed from the field of purine chemistry and biochemistry (Horgan 1978). Most of those early procedures were extremely labor-intensive, time-consuming, imprecise and relatively insensitive (MacDonald and Morris 1985). Several reports have demonstrated the advantages of high performance liquid chromatography (HPLC), for the separation of cytokinins in bioassays, over the more commonly used chromatographic methods of ion-exchange, paper and thin-layer, Sephadex LH20, and GLC (Challice 1975; Kannangara et al. 1978; MacDonald et al. 1981). Unfortunately, HPLC in association with bioassays is still labour-intensive and time consuming. The introduction of mass spectrometric methods in which the cytokinins were converted to volatile derivatives and subjected to gas liquid chromatography (GLC) prior to mass spectrometry (MS) improved precision but not sensitivity (Summons et al.

1979). Detailed reviews about GC-MS and related methods for analysis of cytokinins have pointed out the necessity of several purification methods involving extraction, column separations, thin layer chromatography (TLC), HPLC and GC (Horgan 1978; Palni et al. 1986). The major challenge in the application of this technique is the purification of the complex plant extracts to a less-complex mixture where the mass spectrometric response may specifically correlate with cytokinin content (Hedden 1986; Teller 1994).

Radioimmunoassay (RIA), initially developed to quantify human hormones at physiological tissue concentrations, was adapted to determine cytokinins and became a highly specific and very sensitive analytical method (Weiler 1980). The past thirty years have witnessed a marked growth in the use of specific antibodies in studies of cytokinin biology. Because cytokinins occur in many forms (Fig. 2.3) at relatively small concentrations, analytical procedures with high sensitivity and selectivity are required. Immunoassays were applied to the analysis of cytokinins after it was demonstrated that coupling of nucleosides to proteins gave immunogenic conjugates, which could be used to produce nucleoside-specific antisera (Milstone et al. 1978; Constantinidou et al. 1978). Quantification of cytokinins by immunoanalysis is based upon the competition of cytokinins present in a sample with a known quantity of labeled or immobilized cytokinin for binding to an anti-cytokinin antibody. The degree of competition is measured using either radiolabeled cytokinin (RIA) or enzyme-linked immunosorbent (ELISA) assays. A standard curve based upon the addition of specific quantities of cytokinins to the assay is used to quantify cytokinins present in samples. Since the first report

of an anti-cytokinin antibody by Hacker et al. (1970), antisera and monoclonal antibodies have been used to isolate and quantify endogenous cytokinins from a wide variety of plant tissues (Weiler 1984; Hansen et al. 1988; Saavedra-Soto et al. 1988; Doumas et al. 1989) and microbial sources (Muller et al. 1988; Taller and Wong 1989; Kraigher et al. 1991; Morris et al. 1991; Upadhyaya et al. 1991). As immunoassays have both high specificity and sensitivity with detection limits at the femtomole level, plant crude extracts can be used (Weiler 1984; Weiler and Ziegler 1981; Young 1989; Belding and Young 1989). Other advantages of the immunoassays are that at least one hundred samples can be completed in one day and the assay reproducibility is high with coefficients of variation of triplicate samples of less than 5% (Weiler 1984). Immunoassays have a wide field of application because they allow a much more detailed resolution in time and space of cytokinin levels within whole plants and other plant systems, avoiding or simplifying complicated extraction procedures. However, the potential of immunoassay should not be overestimated and appropriate assay validation should always be considered (Banowetz 1994). Extensive standardization procedures and repetitive dilution of the samples are standard for immunoanalysis and compared to other assays it has low sample capacity (Weiler 1984; Banowetz 1994).

Although the major analytical difficulties in physiological work, namely the rapid and reliable quantification of small amounts of physiologically relevant cytokinins as well as other PGRs in large series of samples, cannot be solved with the GC-MS technique, this analysis undoubtedly will remain as the method of

choice for identification of cytokinin structures, elucidation of new structures in metabolic studies, and validation of immunoassay (Banowetz 1994).

2.3. PGRs in the Rhizosphere

Because much of the chemical information moving from the root to the shoot can be modified as a result of altered root functioning as soil conditions change, it is important to consider that soil microorganisms living in the neighborhood of the roots can produce cytokinins and other PGRs.

The term rhizosphere is the soil region under the immediate influence of plant roots. The root surface itself is recognized as a critical site where interactions between microbes and plants occur and is designated as the rhizoplane (Paul and Clark 1989). Although, it is often functionally or experimentally difficult to distinguish the rhizoplane from the rhizosphere, epidermal and cortical tissues of roots have been shown to harbor different kinds of microorganisms (Rovira, 1991). Microbial ecology of the rhizosphere refers to the study of the interactions of microorganisms with each other, plant roots and the environment surrounding them (Bolton et al. 1993). Microflora that are able to produce PGRs *in vitro* are present in appreciable numbers in the rhizosphere of plants (Barea et al. 1976; Kampert et al. 1975; Arshad and Frankenberger 1993).

Some researchers have subdivided the rhizosphere into the ectorhizosphere or the outer rhizosphere and the endorhizosphere or the inner rhizosphere where invasion and colonization of root cortical cells occurs (Balandreau and Knowles, 1978; Dommergues, 1978). These terms are useful in modeling the rhizosphere,

but most researchers have emphasized that, as the root ages, the cortex and epidermal cells become non-functional and lyse, forming a continuum of soil, root cells and exudates (Kloepper et al. 1992; Lynch 1994). In this thesis, the term rhizosphere will encompass both the rhizosphere (endorhizosphere and ectorhizosphere) and the rhizoplane.

A wide variety of organic compounds of plant origin have been found in the rhizosphere. They have been classified as exudates, secretions, mucilages, mucigel and lysates (Rovira et al. 1979). Sugars, amino compounds, organic acids, fatty acids and sterols, nucleotides, enzymes and PGRs have been detected by several authors who have studied the quantity and composition of the rhizosphere (Curl and Truelove 1986). As much as 40% of the carbon assimilated as photosynthates can be released from roots in a great variety of substances (Lynch and Whipps 1990). Soils differ considerably in their PGR-synthesizing capacity, depending on their fertility status and organic matter content (Stevenson, 1986; Arshad and Frankenberger 1993). Auxins in soil are derived from decomposition of carbonaceous materials from dead and living plant residues (Whipps and Lynch, 1983). The continuous release of root-derived organic carbon compounds in the rhizosphere is called rhizodeposition and stimulates an active rhizosphere microflora. Ratios of colony forming units (cfu) of rhizosphere communities to those of soil communities (R/S ratio) are in the order of 10-50 for bacteria while fungi, protozoa and algae have R/S ratios of 5-10, 1-3 and 1-2, respectively (Paul and Clark, 1989). A dynamic phenomenon occurring in the rhizosphere is that the high population of bacteria in it is usually

accompanied by high faunal activity, especially in those groups that are grazers on the microflora or on roots (Rovira 1991).

The interest in rhizosphere microbiology derives from the ability of the soil microbiota to influence plant growth and vice versa. Suslow (1982) has pointed out that the effects of microorganisms on plant growth are very complex because of the antagonistic and synergistic interactions between different rhizosphere microorganisms. Besides the rhizosphere interactions with the soil, indigenous microflora capable of metabolizing PGRs may affect the net balance of these compounds available for plant uptake. Microbial production of PGRs may benefit the microorganisms involved as well as the plant (Paul and Clark 1989). Likewise, Rossi et al. (1984) reported that auxin- and gibberellin-like components were more abundant in the rhizosphere soil of maize than in non-rhizosphere soil, especially during seedling emergence while the highest amounts of cytokinin-like components were observed during anthesis.

There are numerous microorganisms actively involved in the synthesis of PGRs in pure culture and in soil. Soil isolates, including bacteria, fungi, yeasts, actinomycetes and algae, are capable of synthesizing PGRs which can be estimated by different techniques such as paper chromatography coupled with bioassay, gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-ultraviolet spectrometry (HPLC-UV) and radio and immunoassays (Arshad and Frankenberger 1993).

Van Staden and Dimalla (1976) have observed that the cytokinin activity in acidic soils supporting *Acacia mearnsii* yielded high Z activity while soil

supporting trees in association with mycorrhizal fungi, yielded an activity peak corresponding to ZR. However, it was not determined whether the cytokinins isolated from soils were leached from senescing leaves or were produced by the fungi or other microorganisms.

Ho (1986) observed that phosphatase, nitrate reductase activities and PGR production differed considerably among different isolates of *Pisolithus tinctorius*. Thus, PGR production expressed as micromoles per gram of fungal dry weight was reported in the range of 22.1-271.4 for cytokinins, 56.0-1045.4 for IAA and 5.2-19.3 for gibberellins. Variability in ectomycorrhizal development among isolates of *P. tinctorius* has been reported (Molina 1979; Marx 1981) and was related to PGRs liberated by their mycelia (Navratil and Rochon 1981). However, the significance of these *in vitro* variations to successful inoculation and desired host response needs to be experimentally determined in fungus-host-soil systems.

Allen et al. (1980) have observed changes in PGRs such as IAA and cytokinins in plants infected by vesicular-arbuscular-mycorrhizae (VAM). They found higher cytokinin levels in leaves and roots of grasses in a VAM association, but they concluded that the interaction of PGRs, phosphorus nutrition and photosynthesis in VAM associations was difficult to evaluate.

Although a higher percentage of microorganisms isolated from rhizosphere soil than from root-free soil are capable of synthesizing PGRs, they can influence plant growth only if the released PGR is taken up by the plant and is not metabolized by other microorganisms. For that reason, symbiotic associations

such as mycorrhizal fungi, provide a direct route for PGR uptake by plants, establishing a bridge connecting the plant root with the surrounding soil microhabitats (Azcon-Aguilar and Bago 1994). Although, mycorrhizae are widespread in the plant kingdom, the physiological effects of mycorrhizal PGRs need further study in order to improve their beneficial potential in specific plant associations (Arshad and Frankenberger 1990). There is evidence that changes in the root exudation patterns, PGR balance of the plant and PGR production by rhizosphere microorganisms affect the establishment of mycorrhizal fungi on the root cortex (Barea 1997). The above evidence indicates that microbe-microbe interactions are crucial to the understanding of events that occur at the root-soil interface (Lynch 1990).

2.4. Plant Growth Promoting Rhizobacteria (PGPR).

The soil microflora can promote plant growth by enhancing soil organic matter transformations, mobilizing inorganic nutrients, producing PGRs, acting as antagonists against pathogens and by several other mechanisms (Bolton et al. 1993). Scientists have attempted to alter the microflora of agricultural soils in order to favor plant growth and health. Bacterization is a term that has been used to describe the inoculation of bacterial cultures onto seeds, seedlings and/or vegetatively propagated units (Brown 1974). The subset of rhizosphere bacteria that aggressively colonize roots was termed rhizobacteria (Schroth and Hancock 1982). Root colonization describes an active process, which establishes a permanent relation between bacteria and roots in soil. Bacteria should survive

inoculation onto seeds or into soil, multiply in the spermosphere in response to seed exudates, attach to the root surface and colonize the developing root system in soils containing indigenous microorganisms (Kloepper 1993). Although root colonization and rhizosphere colonization generally are considered synonymous, there is a functional difference between them. Root colonization refers to bacteria that reside or survive outside and inside the roots while rhizosphere colonization describes a larger niche-encompassing root colonization and includes bacteria that are in close proximity to but not necessarily in contact with the roots (Bolton et al. 1993).

Most of the known rhizobacteria belong to Gram-negative genera but the mechanisms by which they colonize roots need further research (Kloepper 1993). The general effects of rhizobacteria on host plants can be deleterious, neutral or beneficial (Bolton et al. 1993). Kloepper and Schroth (1978) have stated that those rhizobacteria that exert beneficial effects on plant growth and/or development should be named plant growth-promoting rhizobacteria or PGPR.

2.4.1. Inoculation Effects of PGPR on Plant Growth and Development

PGPR, such as *Azotobacter*, *Azospirillum* and *Rhizobium* have been widely studied and they can produce direct effects on plant growth because they induce alterations in plant physiology or produce metabolites such as PGRs that directly promote plant growth without interactions with native soil microflora. Evidence showing that *Azotobacter*, *Azospirillum* and *Rhizobium* can induce direct growth promotion is summarized as follows.

Soviet workers have used *Azotobacter* species extensively as biofertilizer (Arshad and Frankenberger 1993) and significant effects were found with several crops when this PGPR was well established within the rhizosphere (Barea and Brown 1974; Hussain et al. 1987). Although other modes of action have been proposed for these associations, a number of publications have indicated that the beneficial effect of *Azotobacter* on plant growth is due to the production of PGRs (Brown 1974; Azcon and Barea 1975; Barea and Brown 1974; Nieto and Frankenberger 1989).

The five species of *Azospirillum* are some of the most studied PGPR (Bashan and Holguin 1997a) and they were isolated from the rhizosphere of different gramineae including forages and grain crops (Magalhaes et al. 1983; Dobereiner and Pedrosa 1987; Reinhold et al. 1987; Khammas et al. 1989). Data from worldwide field experiments accumulated over the past 20 years indicated 60-70 % occurrence of success due to *Azospirillum* inoculation with significant increases in yield in the order of 5-30% (Okon and Labandera 1994). There is evidence for three possible processes or direct effects, which could act independently or in combination, i.e.: nitrogen fixation, improved mineral and water uptake, and production of PGRs (Bashan and Holguin 1997b). With respect to the two first mechanisms, the extensive information available has been compiled elsewhere (Dobereiner and Pedrosa 1987; Kapulnik et al. 1984; Murty and Ladha 1988; Boddey et al. 1986; Sarig et al. 1988; Bashan and Levanony 1990; García de Salamone et al. 1996). Also, it has been reported that the effects of inoculation with *Azospirillum* on root morphology can be mimicked by

applying IAA (Morgenstern and Okon 1987) or mixtures of auxin, GA_3 and kinetin (Hubbell et al. 1979; Tien et al. 1979). Fallik et al. (1989) reported that the inoculation of maize seedlings with *Azospirillum* significantly increased root surface area and inoculated roots were found to contain higher amounts of both free and bound IAA as compared to the control. Indole-3-butyric acid (IBA) was also identified. This was a pioneering paper in the detection of changes in endogenous PGRs after PGPR inoculation.

There is evidence that PGR synthesis is involved in the highly specific *Rhizobium* – legume symbiosis in which as much as the 90% of the plant's requirements for nitrogen are supplied by nitrogen fixation (Puppo and Rigaud 1978; Phillips and Torrey 1972; Morris 1986). Auxin and cytokinin applications to roots in hydroponic media produced morphological changes in the roots, which were similar to those observed on plants inoculated with *Rhizobium* (Skoog et al. 1965; Puppo and Rigaud 1978). Sequeira (1973) showed that *Rhizobium* can stimulate cell division in the cortex and release auxins and cytokinins at the root surface or in the infection threads. Although, the production of active auxin might be limited *in situ*, cytokinin activity was detected in the medium of *Phaseolus vulgaris* plants inoculated with *Rhizobium phaseoli*, but not in medium containing the same amounts of uninoculated roots or rhizobial cells alone (Puppo and Rigaud 1978).

A large body of literature exists on other genera of PGPR that produce beneficial effects on plant growth when applied as inoculants. These PGPR include such genera as *Serratia* (Zhang et al. 1997), *Pseudomonas* (Arshad and

Frankenberger 1993; Kloepper 1993; Young et al. 1990), *Burkholderia* (Pedersen and Reddy 1996), *Agrobacterium*, *Erwinia* (Ryder and McClure 1997) and *Xanthomonas* (De Freitas et al. 1997). Gram-positive strains of PGPR have been reported including *Arthrobacter* (Kloepper et al. 1990) and *Bacillus* (Turner and Blackman 1991; Mariano et al. 1997). Among these, *Pseudomonas* PGPR have received much attention (Schippers et al. 1987; Van Loon et al. 1997; Loper et al. 1997). Many strains of pseudomonads have traits that appear to aid in colonization of seeds and roots such as fast growth rates, motility, chemotaxis to root exudates and ability to catabolize diverse nutrient sources (Kloepper 1993). In recent years, there has been an increasing interest in the use of pseudomonad strains for controlling root diseases caused by a number of fungal pathogens. Although cells of *P. fluorescens* displayed the ability to penetrate the epidermis and colonize intercellular spaces in the outermost root tissues, they do not induce host cell degenerative changes similar to those produced by pathogenic bacteria (Benhamou et al. 1996). Recent reports have shown that *Pseudomonas* PGPR produce an array of antifungal metabolites that induce severe cell disturbances in a number of pathogenic fungi (Dowling and O'Gara 1994). In general, biocontrol bacteria can bring about disease suppression through various mechanisms including competition for nutrients and niches, production of antibiotics and induced systemic resistance (Weller 1988; O'Sullivan and O'Gara 1992; Kloepper 1993; Handelsman and Stabb 1996; Keel and Defago 1997). Attempts to exploit these bacteria as potential biological control agents have recently led to the proposal, that besides their recognized

antifungal properties, they also could elicit plant defense reactions (Tuzun and Kloepper 1995). These involve the coordinated synthesis and accumulation of defense molecules that actively contribute to the restriction of pathogen invasion in the plant tissues (Benhamou et al. 1996).

In response to environmental and health concerns about extended use of pesticides, there is considerable interest in PGPR as an alternative biocontrol approach for use in integrated pest management strategies for crop diseases (Raupach and Kloepper 1997). However, inconsistent performance in the field has delayed commercial development and general acceptance of the use of biocontrol PGPR. Therefore, more insights into the mechanisms that govern the interactions between bacteria, plant and pathogen are needed (Lugtenberg et al. 1994).

Biological control induced by PGPR inoculation produces indirect effects on plant growth and development (Kloepper 1993). Thus the plant response is related to the control or decrease of the pathogen activities rather than to direct growth promotion.

Direct growth promotion by *Pseudomonas* PGPR was first reported by Lifshitz et al. (1987). In this study, it was determined that a strain (*P. putida* GR12-2) belonging to a collection of over 4000 cold-tolerant and nitrogen-fixing pseudomonads (Kloepper et al. 1988) directly promoted root growth of *Brassica campestris* (canola) in the absence of either plant pathogens or deleterious microorganisms. Hong et al. (1991) reported that *P. putida* strain GR12-2 fixed N, produced fluorescent siderophores and synthesized IAA, concluding that any

or all of these mechanisms could contribute to root elongation. Recently, it has been reported that a small number of soil bacteria, including *P. putida* strain GR12-2, contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick 1995). Wild type bacteria promoted root elongation of developing canola seedlings, but the ACC-deaminase mutants of this strain did not. ACC is a precursor of ethylene in plants. These results have been interpreted in terms of a model in which *P. putida* strain GR12-2 might promote root elongation by hydrolyzing some of a plant's ACC, thereby lowering the endogenous ethylene concentration and allowing the roots to grow longer (Glick et al. 1994).

Several authors have reported that specific PGPR strains stimulate plant growth and nodulation of leguminous crops when they are co-inoculated with both *Rhizobium* (Grimes and Mount 1984, Chanway et al. 1989) and *Bradyrhizobium* (Polonenko et al. 1987) in field experiments. Zhang et al. (1997) concluded that the improvement of plant growth, development and physiological activities of soybean seedlings after co-inoculation with *Bradyrhizobium* and certain PGPR strains was due to direct effects of PGPR on overall physiology rather than specific effects on nitrogen fixation. As the co-inoculation effects vary with PGPR and rhizosphere conditions, the mode of action of these nodulating-promoting rhizobacteria needs further research.

A core collection of elite PGPR strains, including *Pseudomonas* and *Serratia* (Kloepper et al. 1988) was screened for PGR production and a relationship between induction of root elongation and production of threshold concentrations

of some cytokinins was observed (Young et al. 1990). However the production of these cytokinins was not studied in the presence of plant tissues.

As the ecology of pseudomonad PGPR is a relatively new research area and the variability of strain characteristics is large, further studies are needed to explain specific plant responses to the inoculation with specific PGPR strains and thus, understand their mode of action.

Although the uptake of several PGRs by plant roots has been demonstrated, it is unknown if PGRs exogenously supplied from soil or by *Pseudomonas* PGPR can modify plant growth. Under sub-optimal environmental conditions plants may not have the capacity to synthesize sufficient endogenous PGRs for optimal growth and microbial PGR production may have an important compensatory role. Thus, the stimulation of microbial biosynthesis of PGRs within the rhizosphere using specific PGPR strains may be an effective approach to improving plant growth and development.

The number of papers concerned with the direct effects on plant growth of the inoculation with PGR-producing *Pseudomonas* PGPR strains has been limited in recent PGPR literature and in the proceedings of the last three PGPR workshops, (Keel et al. 1990; Ryder et al. 1994; Ogoshi et al. 1997). For this reason, direct effects on plant growth of PGPR strains with proven ability to produce PGRs should be further studied.

2.4.2. PGPR and PGR production

The accumulated pool of PGRs in the rhizosphere is a function of production, uptake and breakdown, indicating that the amounts detected are generally less than the amounts that are actually synthesized (De Leij and Lynch 1997). Appreciable accumulation of PGRs in the rhizosphere does not occur because of the heterotrophic activity of the microflora (Lynch 1990). Arshad and Frankenberger (1993) pointed out that the production of PGRs as microbial metabolites in soil has been linked to substrate availability, but the number of influencing factors is high. These authors have also shown that IAA and certain cytokinins can be produced in soil incubated with tryptophan and specific precursors such as adenine and/or isopentenyl alcohol. Bolton et al. (1993) reported tryptophan and adenine could be detected in plant root exudates. Gibberellin-like bioactivity has been detected in several microbial cultures, and it has been suggested that gibberellins, along with other microbial metabolites may affect plant growth and development (Arshad and Frankenberger 1993). However, gibberellins are the least studied PGRs in soil systems. In contrast, more work has been done on ethylene than other PGRs because it is easy to detect. Ethylene has been identified as a common constituent of the soil atmosphere as a result of microbial activity. Primrose (1979) demonstrated that as little as 10 nL L^{-1} of an exogenous application of ethylene can cause a dramatic physiological response in plants and concentrations high enough to affect plant growth are found near the roots and could move rapidly from roots to shoots.

Several authors have reported that *Azotobacter* produces PGRs in pure cultures. Gonzalez-Lopez et al. (1986) estimated the amounts of PGRs produced by *Azotobacter vinelandi* cultured in dialyzed soil media after 96 hours. Auxin activity was equivalent to 0.2-2 μg IAA mL^{-1} in oat coleoptile bioassay, gibberellin activity was 0.8-3.1 μg GA_3 mL^{-1} in a lettuce hypocotyl bioassay and cytokinin-like substance activity was 1.8-4.4 μg mL^{-1} in radish cotyledon bioassay. Also, they observed that the production of auxins, gibberellins and cytokinins was influenced by growth and incubation time. PGRs were released continuously when *Azotobacter* was cultured in N-free medium and dialyzed soil medium. Cytokinin production was about three times higher in dialyzed soil medium than in a defined medium, after 15 days.

Taller and Wong (1989) reported that three cytokinin-active fractions were detected and identified as Z, IPA and IPa in cell-free culture medium following growth of *Azotobacter vinelandi* OP to stationary phase. The total cytokinin activity equivalent was 0.75 μg of kinetin per liter.

Nieto and Frankenberger (1989) also detected cytokinins in cultures filtrates of three *Azotobacter* species. The most prolific producer was *A. chroococcum* and the amount of cytokinin produced was 224 nmol of Z equivalents L^{-1}; when 0.1 m*M* of both adenine and isopentyl alcohol were added to the medium as precursors of cytokinin biosynthesis. These authors observed that the growth of *A. chroococcum* was modified not only by the addition of both cytokinin

precursors, but also by environmental conditions such as pH, carbon sources, N supply, temperature and aeration.

Although *Azospirillum* species are able to produce several PGRs in pure cultures, the amounts were highly variable and strain-specific (Hartmann et al. 1983; Zimmer and Bothe 1988). IAA specifically induced a number of proteins in this bacterium indicating that this bacterium could be used to enhance IAA concentrations in the rhizosphere and thus, to promote growth of the inoculated crops. Muller et al. (1988) stated that the formation of IAA by *Azospirillum* is dependent on tryptophan in the medium, which has been detected in root exudates (Strzelczyk and Potojska-Burdziej 1984). However, at least three routes for IAA biosynthesis were recently demonstrated in this bacterium (Dosselaere et al. 1997).

Many strains of the PGPR *Rhizobium* are capable of producing either IAA (Badenoch-Jones et al. 1982; García-Rodriguez et al. 1984) or cytokinins (Wang et al. 1982) or both (Phillips and Torrey 1972; Newcomb et al. 1977; Upadhyaya et al. 1991) in pure culture at high cell densities. In pure cultures certain fast-growing strains of *Rhizobium* can produce large quantities of the polyamine aminobutylhomospermidine, a tetramine not produced by slowly growing strains, but the physiological effects on roots and other plant parts have not been studied (Galston and Sawhney 1990).

Effective nodulation of *Cajanus cajan* (L). (pigeonpea) with *Rhizobium* strain IC3342 induces a systemic response which results in abnormal shoot development, with symptoms starting 25-30 d after sowing and inoculation

(Letham et al. 1990). Plant symptoms include typical tip bending, followed by hyponasty, curling of leaves, release of apical dominance and proliferation of lateral buds. In grafting experiments, these authors observed that a leaf curl-inducing principle was produced in the root nodules and translocated to the growing shoots through the xylem. A continuous supply was essential for the manifestation of the symptoms. They also observed that the riboside of the cytokinin BAP, supplied via the root system, induced some effects in the shoot, which are characteristic of the leaf curl syndrome, especially release of lateral buds from apical dominance and hyponasty. The main cytokinin metabolites in the xylem exudates of normal nodulated plants and leaf curl plants were the same, but the concentrations of the cytokinins in the latter plants were eight times higher than those in the former. It is particularly significant that plants inoculated with a mutant of IC3342, which did not evoke the leaf curl syndrome, contained cytokinin levels similar to those of normal plants. This is a novel intact plant system to study the role of endogenous cytokinins in shoot development. The metabolism and interaction of cytokinins with other PGRs and their role in plant growth and development are reviewed in section 2.2.

Blackman and Davies (1985) pointed out that a continuous supply of cytokinins from maize roots may be necessary to sustain maximal stomatal opening and that a decrease or an interruption of this supply due to soil drying may act as a signal of reduced root activity. Roots could communicate to the shoot some indications of a perturbation in the soil environment and because the roots are the major source of cytokinins to the plant, a drying or restrictive soil

could reduce leaf cytokinin levels sufficiently to affect stomatal behavior (Zhang and Davies 1989; Zhang and Davies 1991; Tardieu et al. 1992).

In summary, there is enough evidence to support the hypothesis that alterations in plant growth and development in response to inoculation may be due to microbial production of cytokinins. Although there are many reports on cytokinins as endogenous plant metabolites, microbial production of them and their subsequent direct effects on plant growth have received little attention.

CHAPTER 3. MATERIALS AND METHODS

3.1. Culture of Selected PGPR Strains

Table 3.1. describes all the PGPR strains used in this work in order to study direct beneficial effects on plant growth. All the strains were included in pure culture experiments. Only some selected strains were included in further experimental work.

3.1.1. Maintenance of Transposon Insertion Mutants or Transconjugants of *Pseudomonas fluorescens* strain G20-18WT

Pseudomonas fluorescens strain G20-18WT is a PGPR which was isolated from native grass roots on Ellesmere Island, Northwest Territories, Canada (Table 3.1). In early screening, this strain was first identified as a promoter of soybean emergence, nodulation and root elongation. Also it had no phytopathogenic, phytotoxic or deleterious effects on crops such as bean, canola, tomato, cucumber, cotton, corn, wheat, celery, alfalfa, carrot, parsnip and lettuce. In succeeding experiments, this strain was shown to be an excellent colonizer of roots of wheat and soybean, and in pure cultures it produced significant amounts of a cytokinin known as dihydrozeatin riboside (DHZR). This strain was also able to stimulate emergence and improve the growth of wheat (Eco Soil Systems Inc., unpublished).

Table 3.1. Characteristics of PGPR strains used to study direct beneficial effects on plant growth.

PGPR strain	Identification	Origin	Reference
G20-18 Wild Type (WT)	*Pseudomonas fluorescens*	Arctic grass, Ellesmere Island	Eco Soil Systems Inc., unpublished
Mutants of strain G20-18WT			
RIF		Spontaneous rifampicin resistant (100 µg ml-1)	Eco Soil Systems Inc., unpublished
Tn1, Tn2, Tn3, Tn4 (CNT1) [1], Tn5, Tn6, Tn7, Tn8, Tn9, Tn10, Tn11 (CNT2) [1] Tn12, Tn13, Tn14 and Tn15		Transposon insertion mutants	Eco Soil Systems Inc., unpublished
G8-32	*Pseudomonas putida*	Arctic grass, Ellesmere Island	Reddy et al. (1990)
GR12-2	*Pseudomonas putida*	Arctic grass, Ellesmere Island	Lifshitz et al. (1986)
63-28	*Pseudomonas chlororaphis*	Canola field, Manitoba	Reddy et al. (1990)
Ral-3	*Burkholderia cepacia* [2]	Soybean nodules, Alabama	Reddy et al. (1994)

[1] Name given to selected cytokinin negative transconjugants (CNT) after screening for production of dihydrozeatin riboside (DHZR).
[2] Formerly *Pseudomonas cepacia*

Because the ability of *P. fluorescens* strain G20-18WT to increase wheat growth was reported to be variable through years and experimental sites (Eco Soil Systems Inc., unpublished), it was necessary to determine if this strain's ability to promote plant growth was related to cytokinin production. For this reason, transposon mutagenesis to obtain cytokinin-negative transconjugants (CNT) of this PGPR strain was performed, as described by Bayliss et al. (1993). Putative transconjugants (Tn) (1,969 in total) were isolated and conserved in temporary storage. Spontaneous rifampicin resistant mutants had also been previously obtained (Eco Soil Systems Inc., unpublished).

Transconjugant colonies were initially kept on PKS medium (*Pseudomonas* F Agar (PAF), (Difco, Detroit, MI, USA) with 50 $\mu g\ ml^{-1}$ of both kanamycin and streptomycin) at 4°C, with 30 colonies per plate. Each colony was transferred to 2-mL vials containing tryptic soybean broth (TSB) (Difco, Detroit, MI, USA) amended with 20% glycerol using sterile disposable loops. After culturing them for five hours (h) at 30°C, they were stored at -80°C.

3.1.2. Screening of PGPR Strains for Production of Cytokinins

Previously, 110 putative transconjugants had been screened for production of DHZR (Eco Soil Systems Inc., unpublished). No mutant was completely unable to produce this cytokinin. Fifteen out of 110 putative transconjugants (strains Tn1-Tn15 described in Table 3.1.) were screened for their ability to produce DHZR. Ten reproducibly demonstrated an impaired ability to produce DHZR (Eco Soil Systems Inc., unpublished).

PGPR strains, G20-18WT, RIF, G8-32, GR12-2, 63-28 and Ral-3, described in Table 3.1 were also screened for cytokinin production. They were chosen from a core collection of elite PGPR strains partially described by Young et al. (1990) and Reddy et al. (1990, 1994). These strains have been shown to increase root length of canola, cucumber and tomato in controlled conditions. Three of them have also been characterized for their production of DHZR in pure cultures (Reddy et al. 1990 and Eco Soil Systems Inc., unpublished).

PGPR strains were grown on PAF plates from a vial stored at -80 °C. Then they were cultured in TSB for 48 h at 30°C and checked for purity. One small colony of each strain was placed in 9 mL of sterile 0.1 *M* $MgSO_4$ solution to obtain a homogeneous bacterial suspension. One-tenth mL of this suspension was inoculated into sterile disposable 50-mL graduated polypropylene centrifuge tubes, containing 10 mL of minimal medium with 10 g L^{-1} of glucose (MM+Gl) (Appendix A). Three inoculated tubes per strain were put in racks and placed in a 30°C shaker incubator for seven days (d). Samples were taken at 4, 10, 24, 48, 96 and 168 h to assess growth of strains G20-18WT, RIF and selected transposon mutants Tn4, Tn7, Tn8, Tn10, Tn11 (Table 3.1). Bacterial growth was characterized by counting colony-forming units (cfu) on PAF medium using the dilution plate method (Brock et al. 1994). Mean cfu per mL was determined by averaging the log values of populations of three replicated tubes per treatment after 48 h incubation at 30°C. At 168 h, the tubes were centrifuged at 6000 rpm for 20 min. The supernatants were filtered (0.22 µm) into clean tubes and stored

at -20°C until DHZR production was assessed with ELISA test kits as described in section 3.4.1. Experiments were repeated at least three times.

3.1.3. Characterization of *P. fluorescens* Strain G20-18WT and Selected Mutants.

3.1.3.1. Growth curve and time course of cytokinin production

P. fluorescens strains G20-18WT, RIF and transconjugants CNT1 and CNT2, (Table 3.1) were selected for further characterization in pure culture. PGPR strains were grown and checked for purity as described in section 3.1.2. Then, they were cultured at 25°C in 250-mL Erlenmeyer side-armed flasks with 100 mL of MM+Gl medium and shaken at 125 rpm. Each treatment was replicated three times. Culture samples were taken after 4, 10, 24, 30, 48, 72, 96, 168 and 336 h of growth. Growth was assessed by counting cfu mL^{-1} as described in section 3.1.2. At every sampling time, supernatants were obtained by centrifuging 3 mL of culture at 10000 rpm for 20 min (2°C), filtering through Millipore membranes (MILLIPORE, Bedford, MA, USA) of 0.22 µm and storing at -20°C until used. Immunoassays were performed to determine the amounts of the three cytokinins, isopentenyl adenosine (IPA), trans-zeatin ribose (ZR) and DHZR present in supernatants of each strain, as described in section 3.4.1. Further analysis of cytokinins was performed for samples taken after 72, 96, 168, and 336 h of growth as described in sections 3.4.2 and 3.4.3. Experiments were repeated at least three times.

3.1.3.2. Production of indole acetic acid (IAA) and gibberellin-like substances

Samples of pure cultures of *P. fluorescens* strain G20-18WT taken after 3, 4, 7 and 14 d of growth, and samples of strains G20-18WT, RIF, CNT1 and CNT2 taken after 14 d of growth were tested for their ability to produce IAA and gibberellin-like substances in MM+Gl medium. Culture conditions were as described in section 3.1.3.1. Extraction procedures and TLC plates were carried out for these samples as described in sections 3.4.1 and 3.4.2. ELISA tests were performed in order to study the production of IAA-like PGRs, and a lettuce hypocotyl bioassay (Frankland and Wareing 1960) was used to assay gibberellin-like substances.

3.1.3.3. Carbon source utilization

Biolog GN microplates (BIOLOG, Inc., Hayward, CA, USA) were used to compare the ability of strain G20-18WT and mutants RIF, CNT1 and CNT2 to utilize or oxidize a range of carbon (C) sources. Biolog GN microplates contain 95 wells, each with a separate sole C source and a control well without a C source. Respiratory activity reduces a redox-sensitive tetrazolium dye forming purple-insoluble formazan, which is accumulated, inside the active cells (Garland 1996).

Strains were grown for 24 h on Tryptic Soy Agar (TSA) plates but strain RIF was also grown on PAF with 100 $\mu g\ mL^{-1}$ rifampicin (PAF+Rif) plates. Bacterial cells were removed from the agar plates with sterile swabs, avoiding the carry

over of nutrients from the agar medium, and placed into sterile glass tubes with 20 mL of sterile normal saline (0.85 % NaCl) prewarmed to room temperature. Inoculum density was adjusted to within the acceptable turbidity range using turbidity standards supplied by BIOLOG Inc. Suspensions of each strain (150 µL) were added to microplate wells. Plates were kept at 30°C and read after 5, 18, 24, 30, 48 and 72 h of incubation.

Color formation in microplate wells was read visually. All wells visually resembling the control well (A-1) were scored as "negative" and recorded as a zero value. All wells with a noticeable purple color (compared to A-1) were scored as "positive" and recorded as a value of one. Absorbance was also measured at 550 and 620 nm using an EIA plate reader (EAR400 SLT-Lab. Instruments, Australia). Mean absorbance values were obtained for each well. The A-1 well served as the blank and color development data [R-C] were obtained by subtracting the mean absorbance value of each response well (R) from the mean absorbance value of A-1 well (C). Negative values were considered as zero in subsequent data analyses. Average well color development (AWCD) for plates was calculated as the mean of the absorbance values for all 95 response wells per reading time.

3.1.4. Effect of Adenine Addition on Cytokinin Production by *P. fluorescens* Strain G20-18WT

Adenine (Ade) is a precursor in the cytokinin biosynthetic pathway (Taller 1994). The effect of Ade addition on cytokinin production by *P. fluorescens*

strain G20-18WT was tested. Experiments were conducted as described in section 3.1.3.1. using MM+Gl medium, with the addition of sterile aliquots of Ade at 10^{-7}, 10^{-5} and 10^{-3} M. Culture samples were taken after 5, 10, 24, 30, 48, 72, 96 and 168 h of growth. Bacterial growth was assessed by counting cfu mL^{-1} as described in section 3.1.2. Sterile supernatants of samples taken at 96 and 168 h of growth were analyzed for cytokinin production using immunoassays as described in section 3.4.1.

3.1.5. Effect of Wheat Exudate on the Growth of *P. fluorescens* Strain G20-18WT

Exudates of wheat seedlings were obtained by the procedure described in section 3.2.1.1. The effect of wheat exudate on the growth of *P. fluorescens* strain G20-18WT in pure culture was studied. Sterile exudate was added to MM+Gl medium before inoculation. The amounts of wheat exudate added were 0, 1, 5, 10, 15 and 20 mL L^{-1}. Culture samples were taken after 4, 10, 24, 30, 48, 96 and 168 h of growth. Bacterial growth was assessed by counting cfu mL^{-1} as described in section 3.1.2.

3.2. *Pseudomonas* PGPR in Association with Whole Plant Systems

As described in section 2.3.1 of the literature review, seed inoculation with PGPR may increase plant growth but the effects differ with PGPR-plant associations and environmental conditions. The study of two whole plant

systems, wheat and radish, was addressed in this project to elucidate the mode of action of selected *Pseudomonas* PGPR strains.

3.2.1. *Triticum aestivum* (Wheat) cv. Katepwa

Seeds of *Triticum aestivum* cv. Katepwa (SeCan Association, Agriculture & Agri-Food Canada, Winnipeg, MB) were used for all wheat experiments. Three different experimental approaches were used as described below.

3.2.1.1. Growth pouches (GP)

Surface-sterilized wheat seeds were planted in sterile growth pouches (GP) (Vaughan's Seed Co., Downers Grove, IL.). GP were filled with 10 mL of deionized water before autoclaving for 60 min at 121°C. Seeds were surface-sterilized by soaking in 2% sodium hypochlorite solution for 10 min followed by thorough rinsing in sterile water and air drying in a laminar flow-hood overnight. Four seeds were used per pouch with ten pouches per treatment. Three experiments were conducted including *P. fluorescens* strain G20-18WT, mutant RIF and selected transconjugants Tn4 (CNT1), Tn7, Tn8, Tn10, Tn11 (CNT2). Strains were cultured in sterile disposable 50-mL graduated polypropylene centrifuge tubes containing 10 mL of TSB medium in a 30°C shaker incubator for 24 h. Tubes were centrifuged twice at 6000 rpm for 10 min and washed with sterile 0.1 *M* $MgSO_4$ solution. Seeds were immersed in bacterial suspension containing 10^9 cfu mL^{-1} or in sterile 0.1 *M* $MgSO_4$ solution for 3 h before starting the experiments. Bacterial density on inoculated seeds, after soaking, was higher

than $3x10^4$ cfu $seed^{-1}$. GP were kept at room temperature (22-25°C). After 7 d, the GP were slit open and the number of germinated seedlings per GP was recorded. Root and shoot lengths were also determined. The roots of three GP per treatment were carefully removed and placed in 9 mL of sterile 0.1 *M* $MgSO_4$ solution. Suspensions of rhizosphere bacteria were obtained by mixing with a Vortex mixer for 1 min, followed by serial dilution (Brock et al. 1994). Suspensions from root seedlings inoculated with strains G20-18WT, RIF and transconjugants were plated on PAF, PAF+Rif or PKS media, respectively. The dry weight of plants samples was determined by drying at 80°C to constant weight. The mean cfu per g dry weight of root was determined as described in section 3.1.2.

GP filled with 35 mL of deionized water were prepared to collect Katepwa wheat exudate after 15 d of incubation in the same conditions described above. Exudates were collected and sterilized by filtration (0.22 μm). Sterile exudates were stored at -20°C until used as described in section 3.1.5.

3.2.1.2. Greenhouse

PGPR strains and inoculation procedures described in section 3.2.1.1. were used to inoculate Katepwa wheat seed for a greenhouse experiment. Plants grew for 21 d in small pots inside flat trays. Each tray held 48 small pots (25 mL) filled with Perlite (W.R. Grace & Co., Ajax, Ontario, Canada). Each small pot was planted with two seeds at a depth of 1 cm. Three trays per treatment were used. The trays were arranged in a completely randomized design on a plant growth

bench in a greenhouse maintained at 25/15°C with a 16/8 h light/dark cycle. Trays were irrigated with Hoagland's mineral nutrient solution (Hoagland and Arnon 1938) once a week and dechlorinated tap water was used as needed.

Seedling emergence was recorded at 4, 11 and 17 d after planting (d.a.p.). At harvest (21 d.a.p.), root colonization was assessed on seedlings in six small pots per tray as described by Zablotowicz et al. (1992). Shoots of these seedlings were washed under tap water and surface-sterilized by soaking in 70% ethanol for 5 min and rinsing thoroughly with sterile deionized water. Surface-sterilized shoots were aseptically cut in small pieces and 1 g per sample was placed in 9 mL of sterile 0.1 *M* $MgSO_4$ solution. Rhizosphere and shoot suspensions were prepared and plated as described in section 3.2.1.1. All plate media contained 50 $\mu g\ mL^{-1}$ of both cyclohexamide and benomyl. Roots were washed free of Perlite by wet sieving under tap water. Root and shoot biomass was determined by drying the plants at 80 °C to constant weight.

3.2.1.3. Growth chamber

Two replicates experiments were performed in a controlled growth chamber (Conviron, model GR-48, Controlled Environments Co., Canada). The objectives were to determine physiological responses of Katepwa wheat inoculated with *P. fluorescens* strain G20-18WT and to study shoot and root colonization. Long-term climate data (1892-1993) were obtained from the temperature and sunrise-sunset tables of Environment Canada, Saskatoon (52° 10' N; 106° 41' W; 501 m) in order to program the controlled environment chamber according to the

average environmental conditions encountered in the spring. Fig. 3.1A. shows temperature and daylength changes during the experimental period. Fig. 3.1B shows temperature changes during three representative days of the experimental period. Photosynthetic photon flux measured at the top of the canopy, was approximately 300 μmol m^{-2} s^{-1}. Bacterial cultures, inoculation and counting procedures were as previously described in section 3.2.1.1.

Ten control or inoculated seeds were planted in 3.8 L-pots filled with autoclaved Redi-earth (W.R. Grace & Co., Ajax, Ontario, Canada). The chemical properties of autoclaved Redi-earth used in this study were as follows: pH 6.3;EC 1.3 mS cm^{-1}; NO_3-N, 72 μg g^{-1}; P, 17.7 μg g^{-1}; K, 23.9 μg g^{-1}; SO_4-S, 875 μg g^{-1}; Cu, 0.6 μg g^{-1}; Fe, 18.7 μg g^{-1}; Zn, 0.8 μg g^{-1}; Mn, 12.2 μg g^{-1}; B, 0.3 μg g^{-1}. Enumeration of the bacterial population in autoclaved Redi-earth was performed a week after sterilization using the dilution plate method (Brock et al. 1994). Samples were taken from 10 pots and initial bacterial suspensions were prepared using 10 g of Redi-earth in 90 mL of sterile 0.1 M magnesium sulfate. Dilutions were plated on PAF medium containing 50 μg mL^{-1} of both cyclohexamide and benomyl. Experiments were arranged in completely randomized designs with 70 pots. Thirty pots were planted with non-inoculated seed, the other 30 pots with seeds inoculated with G20-18WT. The remaining 10 pots were planted with seeds inoculated with mutant RIF in order to study the

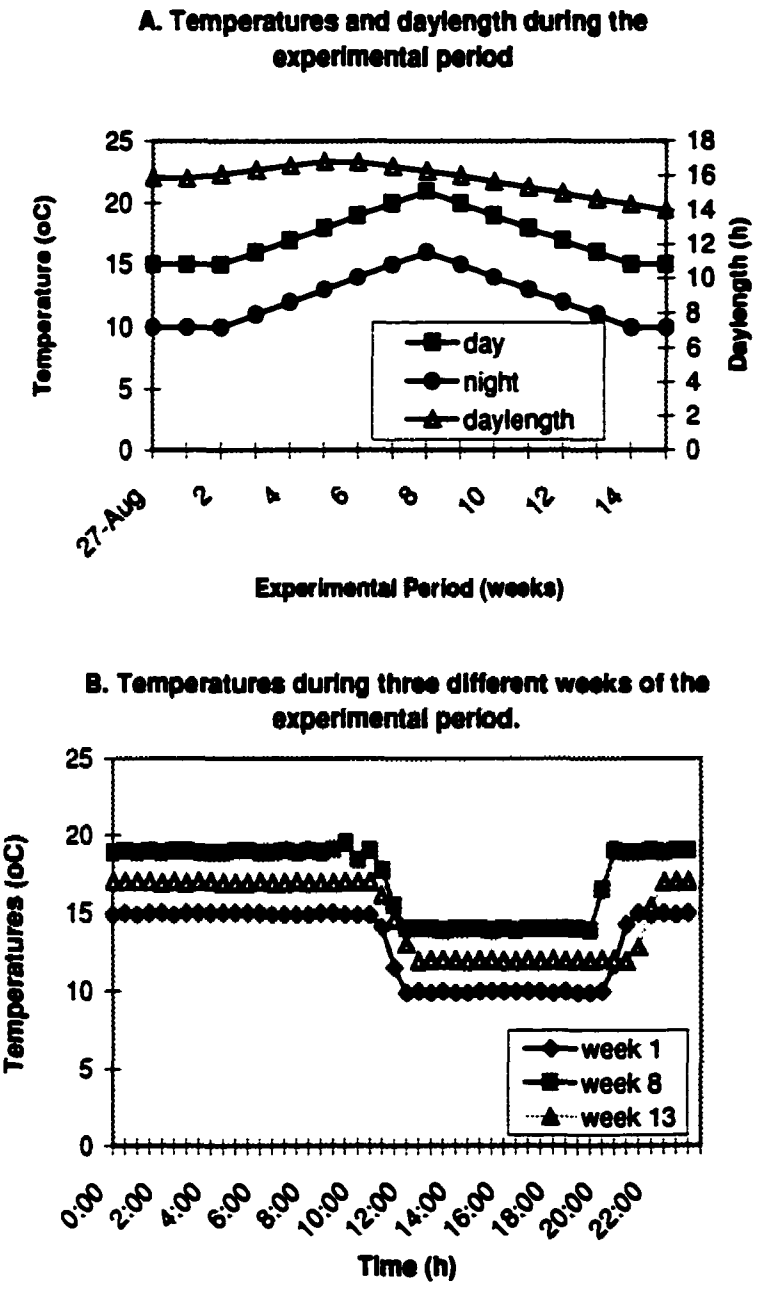


Figure 3.1. Environmental conditions in the growth chamber during the growth of wheat cv. Katepwa inoculated with *P. fluorescens*, strain G20-18WT

capacity of strain G20-18 to colonize the rhizosphere and shoots of Katepwa wheat. Each pot was placed in a deep tray and irrigated with 250 mL per tray of Hoagland's solution once a week and dechlorinated tap water as needed. Pots were covered with small stones autoclaved for 60 min at 121 °C to prevent growth of algae. Pots were rotated periodically.

Emergence was determined 8 d.a.p., and plants were thinned to 2 seedlings per pot. At 15 and 25 d.a.p, five RIF-inoculated pots were assayed for shoot and root colonization as described in section 3.2.1.2. Plant height and leaf number were recorded at weekly intervals between 18 and 46 d.a.p. Plant height was measured from the soil surface to the tip of the longest extended leaf. Leaf numbers included all fully expanded leaves. Tillers were counted at weekly intervals between 25 and 70 d.a.p. The number of visible ears was recorded at 55, 62 and 70 d.a.p. At anthesis (70 d.a.p.) and 20 d later, total leaf area was measured using a non-destructive method as described by Miralles and Slafer (1991). Ten pots per treatment were harvested at 20 d after anthesis. Roots were washed free of Redi-earth as described in section 3.2.1.2 and dry weights of roots, ears and stems were determined and partition indexes were calculated. The remaining 20 pots per treatment were harvested 10 d after physiological maturity was reached (approximately 110 d.a.p.). Stems were excised at ground level and vegetative and reproductive biomass was determined after hand-threshing plant samples dried at 80°C to constant weight.

3.2.2. *Raphanus sativus* (Radish) cv. Cherry Belle

Raphanus sativus (radish) cv Cherry Belle (Northrup King Co., Minneapolis, MN, USA 33440) was chosen as another whole plant system appropriate to study the effects of *Pseudomonas* PGPR inoculation and cytokinin production in the presence of the plant. Three different experimental approaches were used as described below.

3.2.2.1. Growth pouches (GP)

This experimental approach was used with three different objectives, (i.) screening of PGPR strains, (ii.) study of the dose-response of exogenously applied cytokinins and (iii.) PGPR-inoculation response.

GP were autoclaved for 60 min at 121°C after filling with either deionized water, cytokinin solutions or MM+Gl medium as described below. Seeds were surface-sterilized as described in section 3.2.1.1. Five seeds were placed in each pouch with ten GP per treatment. Strains were cultured in sterile disposable 50-mL graduated polypropylene centrifuge tubes containing 10 mL of MM+Gl medium in a 30°C shaker incubator for 48 h. Tubes were centrifuged twice at 6000 rpm for 10 min and washed with 0.1 *M* $MgSO_4$ solution. Unless indicated, the seeds were immersed in bacterial suspensions containing 10^9 cfu mL^{-1} or in sterile 0.1 *M* $MgSO_4$ solution for 3 h before starting the experiments. GP were kept at room temperature ranging between 22-25°C. Dry weight, root and shoot length of seedlings were measured at one or more harvest times. Root

colonization was assessed at zero and harvest times as described in section 3.2.1.1. All the experiments were repeated three times.

Specific methodologies for each experimental set are described as follows.

Pseudomonas PGPR strains were screened to determine which could increase radish growth. Strains, G20-18WT, G8-32, GR12-2, 63-28 and Ral-3 were included (Table 3.1). For this experimental set, GP were filled with 10 mL of deionized water before autoclaving, seeds were inoculated and grown as described above and seedlings were harvested 10 d.a.p.

Three different types of dose-response experiments were performed using exogenously-applied cytokinins. The first type of experiments included only exogenously-applied Z treatments, while the second type of experiments included both exogenously-applied Z and PGPR inoculation treatments. GP, used in first and second types of dose-response experiments, were filled with either 15 mL of deionized water or Z-water solutions ranging between 0.5 and 100 n*M*. The third type of dose-response experiments was performed to compare the effects of exogenously-applied combinations of IPA, Z and DHZR as well as the inoculation with G20-18WT on plant growth. In this case, GP were filled with 40 mL of MM+Gl medium. Experimental trays were gently shaken (100 rpm) to create an aerobic condition for the growth of G20-18WT inside the GP. Filter-sterilized cytokinins were added to the medium at five different concentrations ranging between 0.05-1 nmol GP^{-1}. Each cytokinin concentration was prepared using a ratio of 50:10:40 w/v for IPA, ZR and DHZR, respectively. This ratio was estimated from the concentrations of these components in 7-day

supernatants of GP planted with radish inoculated with G20-18WT as measured in an earlier experiment.

The third set of GP experiments was performed in order to study whether differential responses of radish growth to inoculation were related to the ability of PGPR to produce cytokinins in the radish rhizosphere. The production of cytokinins in pure culture, as well as in contact with radish roots, was measured for the PGPR strains G20-18WT, RIF, CNT1 and CNT2. Non-inoculated GP containing radish plants and inoculated GP with and without radish plants were included in every experiment. GP were prepared using 40 mL of MM+Gl medium and inoculated with 2 mL per GP of bacterial suspensions containing 10^9 cfu mL^{-1}. Bacterial inocula were prepared as previously described. Non-inoculated GP received 2 mL of sterile 0.1 *M* $MgSO_4$ solution. Experimental trays were gently shaken at 100 rpm to create an aerobic condition for the growth of PGPR strains inside GP. Seedlings were harvested at 4 and 7 d.a.p., and bacterial counts were performed as described above. Supernatants of GP media were obtained by centrifugation at 6000 rpm for 20 min, followed by filtering (0.22 µm) and stored at -20°C until used. IAA and cytokinins were estimated by ELISA tests, as described in section 3.4.1.

3.2.2.2. Small pots

Greenhouse experiments were performed in order to study the response of radish to both exogenous applications of cytokinins (Z and DHZR) and PGPR

inoculation. Some experiments were performed as a combination of exogenous applications of a particular cytokinin with inoculation treatments.

Cytokinin concentrations were 0.5, 5, 10, 50 and 100 n*M*, in initial experiments and between 0.5 and 10 n*M*, in the second group of experiments. Finally, experiments were conducted with Z only at concentrations ranging between 0.05-1 n*M*.

Pseudomonas PGPR strains described in section 3.2.2.1 were tested first. In succeeding experiments, a reduced number of strains was included. Finally, only strain G20-18WT and three selected mutants (described in section 3.1.3.1) were included in the remaining experimental work.

Methods used for strain culture, seed inoculation and bacterial counting were described in section 3.2.2.1. The number of cfu on the seeds was determined as described by Zablotowicz et al. (1992). Two seeds were planted in each small pot. Plants grew for 15-18 d in 25-mL pots inside flat trays. Each tray held 48 small pots filled with Perlite. The trays were arranged in a completely randomized design on a plant growth bench in a greenhouse maintained at 25/15°C with a 16/8 h light/dark cycle. Hoagland's solution at 1/4 strength was added to the trays as needed, and was used as the basis for the cytokinin watering solutions. Cytokinins (Z and DHZR) were exogenously supplied to radish plants on a daily basis. Each treatment had three replicated trays. All experiments were repeated three times.

Colonization studies and comparisons between G20-18WT and selected mutants RIF, CNT1 and CNT2 were performed in order to understand the

relationship between strain G20-18WT and radish plants better. In these studies, bacteria were counted at 7 and 15 d in order to study root colonization and survival.

Chlorophyll units were measured on both cotyledons and most expanded leaves of radish before harvesting the plants using a portable chlorophyll meter (MINOLTA Co., Ltd., Japan).

Germination was monitored every day for 3 d after setting up the experiments. Root and shoot biomasses were determined by drying the plants at 80 °C to constant weight. Roots were also analyzed to determine their relative root surface area (RRSA), using the gravimetric method described by Carley and Watson (1966). Root samples were immersed in a water:calcium nitrate solution (1:6, v/v). Initial and final weights of the solution were recorded and used to calculate the relative root-surface of the samples.

Cytokinins in shoot and roots were extracted using the method described by Weiler (1980) and immunoassays were conducted as described in section 3.4.1.

3.2.2.3. Large pots

A greenhouse experiment was performed in order to determine the response of radish cv. Cherry Belle to inoculation with PGPR strains as described in section 3.2.2.1. Strains were cultured as described in section 3.2.1.1. Seed treatments and bacterial counts were as described in section 3.2.2.2. Plants grew for 30 d in 3.8 L-pots that were filled with Perlite. Each pot was placed in a deep tray to allow watering and the addition of 1/4 strength Hoagland's solution from

below. Each pot was planted with five seeds and after 5 d thinned to three seedlings per pot. Pots were covered with small stones autoclaved for 60 min at 121 °C to prevent growth of algae. Pots were arranged in a complete randomized design with 13 replicate pots per treatment and placed on a plant growth bench in a greenhouse maintained at 25/15°C with a 16/8 h light/dark cycle. Pots were rotated periodically.

Bacterial counts of rhizosphere samples were performed 5 d.a.p. as described by Zablotowicz et al. (1992). Chlorophyll units were measured on cotyledons and most expanded leaves 20 d.a.p. using a portable chlorophyll meter as described previously. Biomass of tubers, roots and shoots was determined by drying the plants at 80 °C to constant weight. The fresh weight of tubers was determined gravimetrically.

3.3. Tobacco-Callus Bioassay (TCB)

Tobacco tissues of *Nicotiana tabacum*, var. Xanthi were used in this study. Initially, tobacco callus was obtained from the Plant Biotechnology Institute at the National Research Council of Canada (PBI-NRC). Fresh pith was used to obtain callus, and continuously subcultured callus was used for every experiment. The callus was maintained by transferring every 21 d to new MS24-2 medium, which contained 2 mg L^{-1} of both BAP and 2,4-D (Murashige and Skoog 1962). This medium was named Standard BAP or SD-BAP. Small pieces of white callus ranging between 40-60 mg from 21-day stock callus were used for all experiments. These pieces were placed in groups of three or six on each culture

plate. Plates had a diameter of 100 mm and height of 25 mm and each contained 25 mL of medium. Each treatment had at least three replicated plates. All the experiments were repeated at least twice and run in the same conditions on light tables placed in the lab at room temperatures ranging between 22-25 °C and light/dark periods of 16/8 h. Plates were rotated every 3 d. Unless indicated, calli were harvested at 21 d, and fresh weights were recorded. Fresh weight increases (FWI) of calli were calculated as the difference between initial and final fresh weights of callus.

3.3.1. Cytokinin-Dose-Response Standardization Experiments

The TCB was adapted to estimate the amounts of cytokinin-like compounds produced by PGPR strains. Medium SD-BAP was modified to increase the sensitivity of callus tissue to cytokinins. Table 3.2 describes the TCB experiments performed to determine the growth conditions required to obtain callus, which was very sensitive to cytokinins. It also describes the conditions of stock callus cultures used to perform the experiments. Concentrations of Z and DHZR, added to MS24-2 media, ranged between 0.5 and 100 nmol L^{-1} in these standardization experiments. Afterwards experiments using Z concentrations lower than 0.5 n*M* were also performed on plates with [SD+0] and [0+0] media using stock callus grown on the same and different media.

For all the Z experiments described in Table 3.2, the 0.5 n*M* Z treatment was replicated twice in order to determine the variability in the response of callus

Table 3.2. Description of TCB standardization experiments and MS24-2 media used to determine stock and culture conditions required to obtain callus sensitive to cytokinins.

A. Z Cultures			
2,4-D concentration	BAP concentration	Number of Steps on the same type of medium	Number of Experiments Performed
SD [1]	SD [2]	> 5	7
0	0	1	6
0	0	2	2
SD	0	1	7
SD	0	2	4
SD	0	3	4
SD	0	4	4
B. DHZR Cultures			
2,4-D concentration	BAP concentration	Number of Steps on the same type of medium	Number of Experiments Performed
SD	SD	> 5	3
0	0	1	2
SD	0	1	4
SD	0	3	2

Experiments described included cytokinin concentrations ranging between 0.5-100 nmol L^{-1}.
Concentrations of 2-4D and BAP define the type of MS24-2 medium used.
[1] Indicate standard concentration of 2,4-D, (2 mg L^{-1}).
[2] Indicate standard concentration of BAP, (2 mg L^{-1}).

growth to the addition of Z to the culture medium before or after autoclaving. Based on the results described in Fig. 4.33, filter-sterilized cytokinins were added into media of all succeeding TCB experiments. Standard curves were obtained as described in section 3.5.

3.3.2. Time-Course of Cytokinin Activity in Supernatants of PGPR Strains

P. fluorescens strain G20-18WT and mutants RIF, CNT1 and CNT2 (Table 3.1.) were cultured and filter-sterilized supernatants of the samples were obtained and stored at -20°C until used as described in sections 3.1.3 and 3.1.4. A day before setting up the experiments, 1-mL aliquots of sterile supernatants were added together with melted media [SD+0] or [0+0] (as described in Table 3.2.) into culture plates. Control plates were prepared using 1 mL of sterile 0.1 *M* $MgSO_4$ solution. The same procedure was used to prepare plates with Z concentrations lower than 1 n*M* in order to obtain standard curves as described in section 3.5. After harvest, FWI was calculated as described previously. FWI of the samples was compared to FWI observed for a known concentration of Z on the standard curve. The amounts of cytokinin-like compounds present in the samples were expressed in terms of the concentrations of Z equivalents (ZE) present in supernatants of PGPR strains as estimated from the standard curves.

3.3.3. Effects of Selected *Pseudomonas* PGPR Strains on Tobacco Callus Growth

In all the TCB experiments three types of culture plates were used. These were (i.) tobacco callus control plates (T), (ii.) bacterial control plates (B) and (iii.) plates with tobacco callus and bacteria grown together (T+B). The letter B was replaced by the PGPR strain designation in reported data. As described in section 3.3.2, Z plates were simultaneously cultured for all the experiments, and

ZE present in agar samples was estimated from standard curves. PGPR strains were grown on PAF plates from a vial stored at -80 °C. They were then grown in TSB for 24 h at 30 °C and checked for purity. Strains were cultured in sterile disposable 50-mL graduated polypropylene centrifuge tubes, containing 10 mL of MM+Gl medium and grown in a 30°C shaker incubator for 48 h. Then the cultures were centrifuged twice at 6000 rpm for 10 min and washed with sterile 0.1 *M* $MgSO_4$ solution. Bacterial suspensions were adjusted to a concentration of 10^9 cfu mL^{-1}. Ten µL of PGPR suspensions were streaked onto agar plates at a distance of 2.5 cm from three callus pieces placed at equal distances down the center of the plate. There was no contact between bacteria and tobacco callus. Control and inoculated plates were incubated for 72 h at 30 °C before callus was added. Unless indicated, calli were harvested at 7, 14 and 21 d. Streaks of bacterial growth on the culture plates were carefully removed and placed in 9 mL of sterile 0.1 *M* $MgSO_4$ solution. Bacterial counts were performed on PAF plates as described in section 3.2.1.1. Amounts of cytokinins IPA, ZR and DHZR present in agar samples were estimated using immunoassays as described in section 3.4.1.

3.3.3.1. Interaction between medium composition and presence of *P. fluorescens* strain G20-18WT in TCB plates.

The objective of this study was to determine the effect of medium composition on bacterial survival during the bioassay for the detection of cytokinin-like compounds produced in the presence of tobacco tissue. A series of

TCB experiments was performed using strain G20-18WT with different nutrient sources added to the [0+0] medium. The latter was amended with the following components: 10 g Glucose L^{-1} and combinations of equal concentrations of Bacto Peptone # 3 and Bacto Tryptone in concentrations ranging between 0.02 and 1 g L^{-1}.

3.3.3.2. Detection of cytokinin-like compounds produced by certain *Pseudomonas* PGPR strains in the presence of tobacco callus.

Two sets of TCB experiments were performed to detect cytokinin-like compounds produced by PGPR strains in the presence of tobacco callus. The first set of these TCB experiments was performed on media [SD+0] and [0+0], using stock callus grown one step on medium [SD+0]. *Pseudomonas* PGPR strains G20-18WT, G8-32 and 63-28, previously described in Table 3.1.1 were used. Bacterial counts were only performed at time zero. Calli were harvested after 21 d. FWI and ZE were calculated as described above.

The second set of TCB experiments used medium [0+0] and stock callus grown one step on medium [SD+0]. It was performed using *P. fluorescens* strain G20-18WT (WT) and its mutants RIF, CNT1 and CNT2. Extracts of 21-day samples of T, B and T+B plates were obtained, and the amounts of IAA and gibberellin-like substances were analyzed as described in sections 3.4.1 and 3.4.2. ELISA tests were performed to determine the production of IAA-like PGRs. Gibberellin-like substances were assayed with lettuce hypocotyls by the method described by Frankland and Wareing (1960).

3.4. Analytical Techniques

3.4.1. Immunoanalysis

All cytokinins, IAA and ELISA test kits were purchased from Sigma (Idetek, San Bruno, CA, USA). Samples were obtained from pure cultures of PGPR strains, the rhizosphere of radish plants growing in GP, roots and shoots of radish plants growing in small pots, and agar media of TCB experiments as described previously.

ELISA tests were performed according to kit instructions. Cytokinins and IAA stock solutions (10 m*M*) were prepared in absolute methanol and stored at 4°C. Dilutions were made in glass tubes to obtain standard concentrations for dose-response curves. Cytokinin concentrations were 0.005, 0.05, 0.1, 0.5, 1, 5 and 100 pmoles $(0.1\ mL)^{-1}$. IAA concentrations were 1, 5, 20, 50, 200, 500 and 1000 pmoles $(0.1\ mL)^{-1}$. Samples were diluted as needed in order to obtain accurate estimates within the ranges of standard concentrations. After incubation, the absorbance was read at 405 nm using an ELISA plate reader (EAR400 SLT-Lab. Instruments, Australia). PGR concentrations in samples were estimated from standard curves obtained using absorbance values and standard concentrations as described in section 3.5. For samples from bacterial cultures, GP and radish plants the diluent was MM+Gl medium, the same medium in which the strains were grown for most of the experiments. Samples from bacterial cultures and GP rhizospheres were obtained as described in sections 3.1.3.1 and 3.2.2.1, respectively. Cytokinins in radish plants were extracted as described by Weiler (1980). For TCB samples, the diluent was an

extract obtained using a ratio of 1.5 g of agar medium [0+0] in 5 mL of 80% methanol. These conditions were also used for sample extractions. The pH of the diluent was adjusted to 7.5 with 1 *N* KOH. Agar samples were taken between the bacterial streak and the tobacco callus. Control plates with only bacteria or tobacco callus were sampled similarly. The weight of each agar sample was recorded. Extractions were made in glass tubes placed in racks on a shaker at 4°C in the dark for 48 h. Extracts were then filtered (0.22 µm) and stored at -20°C until used.

3.4.2. Thin Layer Chromatography (TLC)

Further analysis of the PGR composition was performed for pure culture samples of *P. fluorescens* strain G20-18WT taken after 3, 4, 7 and 14 d of growth, and for samples of strains G20-18WT, RIF, CNT1 and CNT2 taken after 14 d of growth. Agar samples of T, WT and T+WT plates of TCB experiments were also further analyzed. Samples were obtained as described above in sections 3.1.3.1, 3.1.3.2, 3.3.3.2 and 3.4.1. One purpose for these analyses was to study the production of IAA and gibberellin-like substances. Another objective was to clean and prepare the samples to confirm the presence of the three cytokinins (IPA, ZR, DHZR) analyzed by ELISA tests using the HPLC-MS technique described in section 3.4.3.

Further extractions of the samples were performed as described by Tien et al. (1979). A control mixture solution containing 1 m*M* IPA; 0.5 m*M* Z; 1 m*M* IAA, and 10 m*M* gibberellic acid (GA_3) was extracted simultaneously with the samples.

Pooled extracts were concentrated in the aqueous phase under reduced pressured on a rotary evaporator and stored at -20 °C until used. Twenty μL of extract samples and authentic compound solutions (1m*M*) were co-chromatographed onto 500 μm-thick silica gel-coated plates (20 x 20 cm, Merck) containing a fluorescent indicator. Chromatography was carried out in *n* -butanol:water:14N ammonia (60:20:10, v/v) to separate cytokinins in *n* -butanol fractions as described by Parker and Letham, (1973). Authentic cytokinin-like substances were Ade, IPA, Isopentenyl Adenine (IPa); Z; ZR; dihydrozeatin (DHZ); DHZR; Zeatin-7-glucoside (Z7G); Zeatin-O-glucoside (ZOG); Adenosine-5'-monophosphate (AMP) and Zeatin Riboside-5'-monophosphate (ZRNT). Separation of indole and gibberellin-like compounds in ethyl acetate fractions was carried out in chloroform:ethyl acetate:formic acid (50:20:10, v/v) as described by Tien et al. (1979) including IAA, GA_3 and $GA_{4/7}$ as authentic compounds. TLC was carried out at least three times for each sample. The chromatograms were thoroughly dried and observed under 254 nm UV light and clearly separated spots were marked. R_F was calculated for authentic compounds as described by Stahl (1969). The chromatograms were cut transversely into two or more ranges of R_F. These sections were eluted separately. Spots in TLC plates for cytokinin separation were eluted with 9 mL of absolute methanol or ethanol:water (1:1, v/v). Spots in TLC plates for gibberellin-like substances were eluted with 9 mL of ethyl acetate. All elution extracts were filtered through 0.22 μm methanol-resistant membrane filters (MILLIPORE, Bedford, MA, U.S.A) and dried in a Speed-Vac (Savant Inc.). Further analysis of gibberellins was

performed using a lettuce hypocotyl bioassay (Frankland and Wareing, 1960). Dose-response curves using pure GA_3 ranging between 0.01 and 100 mg L^{-1} were obtained for each bioassay, as described in section 3.5.

3.4.3. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

Cytokinins fractionated were further analyzed on TLC plates from pure culture samples as described in section 3.4.2. to confirm qualitatively the presence of the cytokinins IPA, ZR and DHZR. These cytokinins were quantitatively analyzed using the ELISA tests as described in section 3.4.1. This study was performed at the Mass Spectrometry Laboratory of PBI-NRC as described below.

Dried samples were made up to 100 µl with acetonitrile:water (50:50, v/v), and filtered through silanized glass wool packed capillaries to remove any particulates and were transferred into injector vials. HPLC was done in isocratic mode with methanol:water (90:10, v/v) with 5 m*M* ammonium acetate. Multiple reaction monitoring (MRM) was used for detection of the HPLC effluent. Each of the three cytokinins was monitored with diagnostic transition MRM of the $(M+H)^+$ ion losing a $C_5H_8O_4$ as described by Prinsen et al. (1995). The equipment used was a Hewlett-Packard hp1100 binary HPLC equipped with a made in house C18 micro column (10 cm x 0.32 mm i.d.-5 µm packing). Acurate microflow rates were maintained using the AcuRATE (by LC Packings) flow splitter. The capillary column was interfaced with a VG

Micromass Quattro LC mass spectrometer via the "Z-spray" source to obtain electrospray spectra.

3.5. Statistical Analyses

Dose-response curves were generated using regression analysis (Excel, Microsoft Seattle, WA) for every ELISA assay, lettuce hypocotyl bioassay and TCB experiment performed.

Biolog data were analyzed using principal component analysis (PCA). PCA is commonly used to reduce complex multidimensional data into a smaller number of interpretable variables or principal components that represent a subset of the original variables. PCA was performed using Statistix^{R} 4.1. (Analytical Software, Tallahassee, FL, USA).

Data obtained from individual experiments using pure cultures, GP, TCB and greenhouse and growth chamber approaches were subjected to analysis of variance and comparison of means and the interaction between experiments and treatments were tested for each set of experiments using Statistix^{R} 4.1. Analysis of variance indicated that there were no significant interactions between experiments when means of several experiments were calculated.

General Lineal Model Procedures (GLMP) from the SAS statistics package (SAS Institute Inc., Cary, USA) were also used for TCB standardization experiments described in Table 3.2.

CHAPTER 4. RESULTS

4.1. Cultures of Selected PGPR Strains.

4.1.1. Screening of PGPR for Cytokinin Production

The five PGPR strains included in this study produced significantly different amounts of DHZR after growing 7 d in tubes containing MM+Gl medium (P =0.05) (Table 4.1.). Strain Ral 3 produced the lowest amount of the cytokinin DHZR and strains G20-18WT and G8-32 produced the highest amounts. *P. putida* strain GR12-2 and *P. chlororaphis* strain 63-28 produced similar amounts of DHZR which were significantly less than those produced by

Table 4.1. Number of bacteria and amount of DHZR produced per mL and cfu by five PGPR strains in pure cultures.

PGPR strains	Number of Bacteria Log [cfu mL^{-1}] (1)	Production of DZHR	
		pmol mL^{-1}	pmol $(10^{10}$ cfu$)^{-1}$
G20-18WT	9.53 a (2)	0.183 a	5.42 a
G8-32	9.35 a	0.127 a	5.69 a
GR12-2	9.46 a	0.043 b	1.49 c
63-28	9.28 a	0.048 b	2.51 bc
Ral 3	9.11 a	0.005 c	0.39 d

(1) Logarithm of colony forming units (cfu) per milliliter after 7 d of growth.

(2) Means followed by the same letter/s are not different as determined by Tukey's test at the rejection level $P = 0.05$.

G20-18WT and G8-32 (P=0.05). The concentration of DHZR was also expressed as pmol per 10^{10} cfu and there were significant differences among strains (P=0.05) (Table 4.1). Strains G20-18WT, G8-32 and 63-28 produced the highest amounts of DHZR and were selected to study the effect of their cytokinin production on tobacco callus grown as described in sections 3.3.3.2 and 4.3.3.2.

The quantities of DHZR produced by strain G20-18WT, mutant RIF and fifteen transposon insertion mutants following growth for 7 d in tubes containing 10 mL MM+Gl medium are shown in Fig 4.1. This screening was carried out to select cytokinin-negative transconjugant (CNT) mutants in order to study the relationship between cytokinin production and plant growth promotion by strain G20-18WT. Strains RIF and G20-18WT produced similar amounts of DHZR. Except for mutants Tn2 and Tn14, all the transconjugants produced lower amounts of DHZR than G20-18WT. However, transconjugants Tn4, Tn7, Tn8, Tn10 and Tn11 were selected on the basis of their reduced production of DHZR and were included in further experimental work. Except for mutant Tn10, all the strains showed similar bacterial numbers and generation times but DHZR production per cfu varied (Table 4.2.). Transconjugants Tn4 and Tn11 produced the lowest amounts of DHZR, which were significantly less than amounts produced by strain G20-18WT (P =0.05). Although DHZR production by these two mutants could be detected by ELISA, it was evident that these strains had an impaired ability to produce cytokinins. For this reason, they were called CNT mutants and named CNT1 and CNT2, respectively as described in section 3.

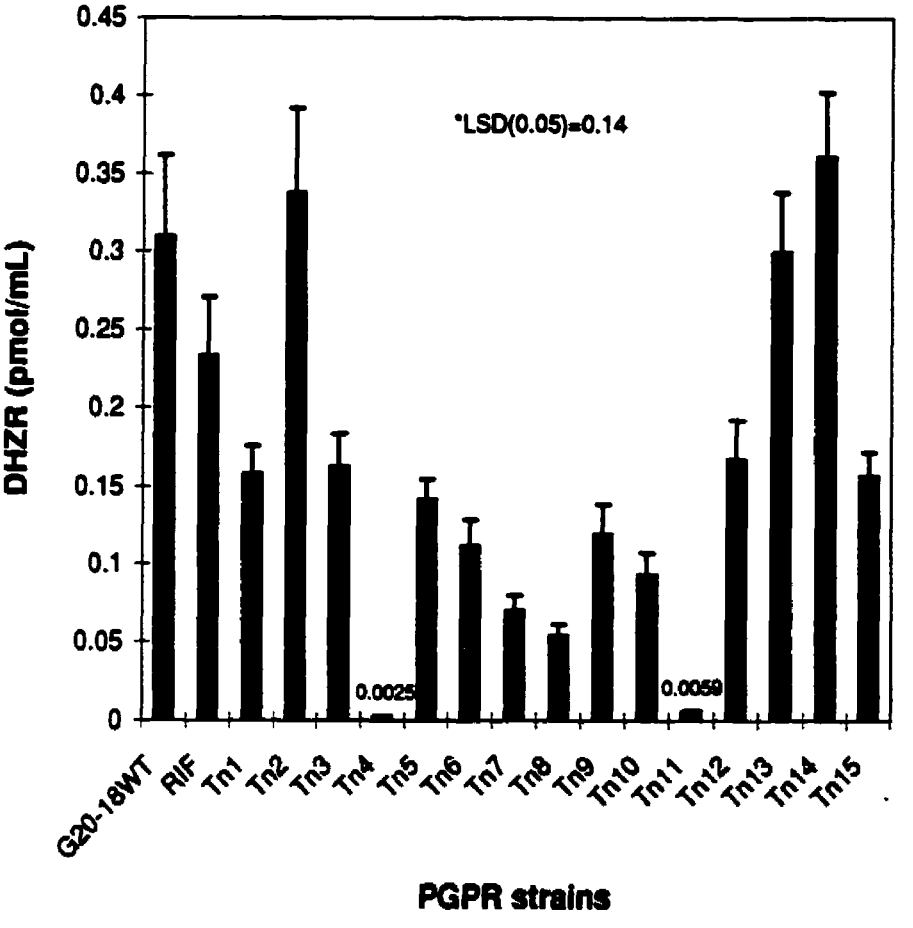


Figure 4.1. DHZR production by *P. fluorescens* strain G20-18WT, mutant RIF and fifteen transposon insertion mutants grown in pure cultures.
Bars are the means of five experiments with three replicated tubes. Vertical lines are standard errors of the means. Means for Tn4 and Tn11 were written on their respective bars because their values were very low.
*LSD, Least significant difference at the rejection level P =0.05 equal to 0.14 pmol mL^{-1}.

Table 4.2. Generation times, total number of bacteria per mL and amounts of DHZR produced by *P. fluorescens* strain G20-18WT and selected mutants in pure cultures.

PGPR strains	Generation Times[1] (h)	Number of Bacteria Log [cfu mL]$^{-1}$[2]	Production of DZHR pmol (10^{10} cfu)$^{-1}$[3]
G20-18WT	2.16 b[4]	9.53 a	9.11 a
RIF	2.10 b	9.45 a	8.27 a
Tn4 (CNT1)	2.02 b	9.97 a	0.03 d
Tn7	2.04 b	9.98 a	0.73 c
Tn8	2.23 b	9.68 a	1.12 c
Tn10	2.64 a	9.46 a	3.22 b
Tn11 (CNT2)	2.11 b	9.28 a	0.31 c
P	0.01	0.05	0.05

[1] Generation times calculated for the exponential phase between 10 and 48 h of growth.
[2] Logarithm of colony forming units (cfu) per milliliter after 7 d of growth.
[3] Amounts of DHZR produced after 7 d of growth in MM+Gl.
[4] Means followed by the same letter did not differ significantly as determined by Tukey's test at the indicated rejection levels.

4.1.2. Characterization of *P. fluorescens* Strain G20-18WT and Selected Mutants.

4.1.2.1.Growth curve and time course of cytokinin production

Fig. 4.2 shows the growth curves and time course of cytokinin production by *P. fluorescens* strain G20-18WT, RIF mutant and transconjugants CNT1 and CNT2 growing in MM+Gl for 14 d. There were no significant differences among strains in the mean generation times or numbers of colony forming units during the stationary phase of growth (72-240 h) (P =0.05). After 240 h the death phase began because bacterial numbers were significantly higher than at 336 h (P=0.05).

There were significant differences in the production of [IPA+ZR+DHZR] by

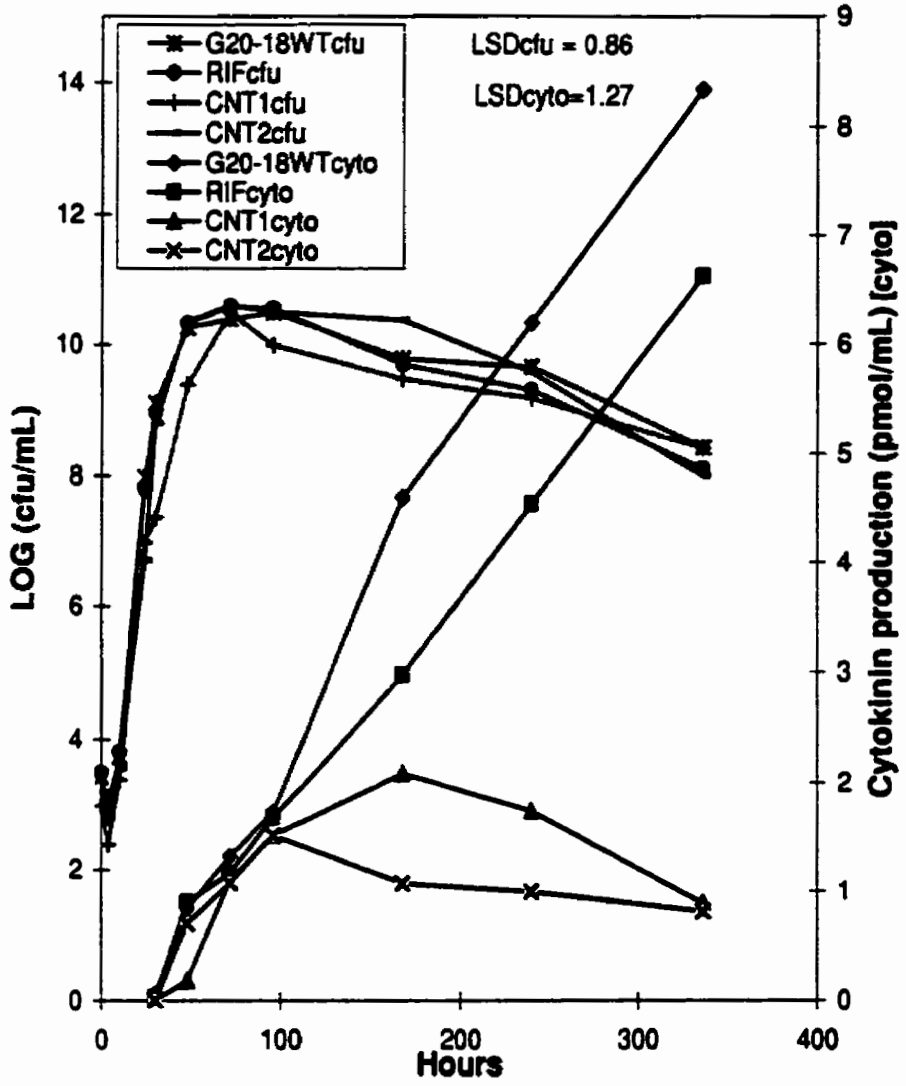


Figure 4.2. Growth curve and time-course of cytokinin production [IPA+ZR+DHZR] by *P. fluorescens* strain G20-18WT and selected mutants growing in pure cultures.

Data are means of five experiments with three replicated flasks. LSD, Least significant difference at the rejection level P =0.05 for cfu equal to 0.86 and for cytokinins (cyto) equal to 1.27 pmol mL^{-1}.

these strains (Fig. 4.2). Cytokinin production was detected after 30 h of growth only for G20-18WT and RIF strains and estimated to be 0.060 and 0.027 pmol mL^{-1}, respectively. No differences among strains were observed between 30 and 96 h. Cytokinin production by CNT2 ceased after 96 h, while the other three strains continued to produce cytokinin at different rates. After 168 h the concentrations of [IPA+ZR+DHZR] were 4.6, 2.98, 2.11 and 1.07 pmol mL^{-1} for G20-18WT, RIF, CNT1 and CNT2, respectively. Strain G20-18WT produced significantly higher amounts of [IPA+ZR+DHZR] than the three mutants from 168 h until 336 h (P=0.05). Cytokinin concentrations in CNT1 and CNT2 cultures after 240 h and 336 h were similar but significantly lower than the amounts of [IPA+ZR+DHZR] produced by strain RIF (P=0.05). Cytokinin production by strains G20-18WT and RIF was still increasing between 240 h and 336 h when the death phase had begun. The opposite was observed for mutants CNT1 and CNT2.

IPA production began after 30 h of growth for strains G20-18WT and RIF but it was not detected until 48 h for mutants CNT1 and CNT2 (Fig. 4.3). No differences in the production of this cytokinin were observed among strains between 30 and 96 h. The amounts of IPA produced by strain RIF were significantly lower than those for strain G20-18WT at every sampling time after 96 h (P=0.05). Strain G20-18WT always produced significantly higher amounts of IPA than mutants CNT1 and CNT2, after 96 h (P=0.05). Strain RIF only produced significantly higher amounts of IPA than mutants CNT1 and CNT2, after 240 and 336 h, respectively (P=0.05). ZR production by strain G20-18WT

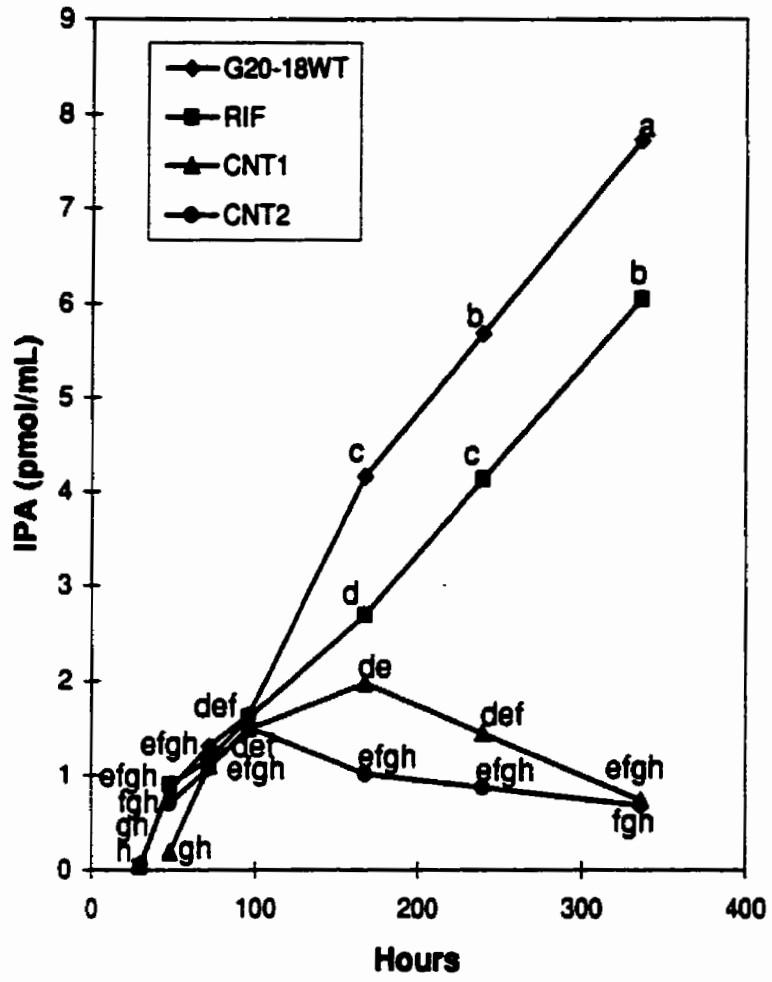


Figure 4.3. Time-course of isopenthenyl adenosine (IPA) production by *P. fluorescens* strain G20-18WT and selected mutants growing in pure cultures.

Means followed by the same letter/s are not significantly different as determined by Tukey's test at the rejection level of $P = 0.05$.

and selected mutants was detected only after 96 h, but significant differences among strains were only observed after 168 h (Fig. 4.4). Strains G20-18WT and RIF always produced significantly (P=0.05) higher amounts of ZR than mutants CNT1 and CNT2 after 168 h. However, the amounts produced by strain G20-18WT were always significantly higher than the amounts produced by the RIF mutant (P=0.05).

DHZR production began after 48 h of growth for strains G20-18WT and RIF but it was not detected until 72 h for mutants CNT1 and CNT2 (Fig. 4.5). No significant differences in the production of this cytokinin among strains were observed between 48 and 96 h. At 168 h, strain G20-18WT produced significantly higher amounts of DHZR than mutants CNT1 and CNT2 but not RIF (P=0.05). At 168 h, G20-18WT produced significantly more DHZR than the RIF strain (P=0.05).

Differences in the proportion of each cytokinin produced relative to the total [IPA+ZR+DHZR] were observed among the strains (Table 4.3).

The percentage IPA was always higher than 90% except in the mutants CNT1 and CNT2 at 240 and 336 h of culture. Percentages of ZR and DHZR produced by G20-18WT and RIF were similar throughout the growth period and ranged between 2.16 and 5.56% of [IPA+ZR+DHZR] produced. The mutants CNT1 and CNT2 accumulated increasing proportions of ZR and DHZR with time.

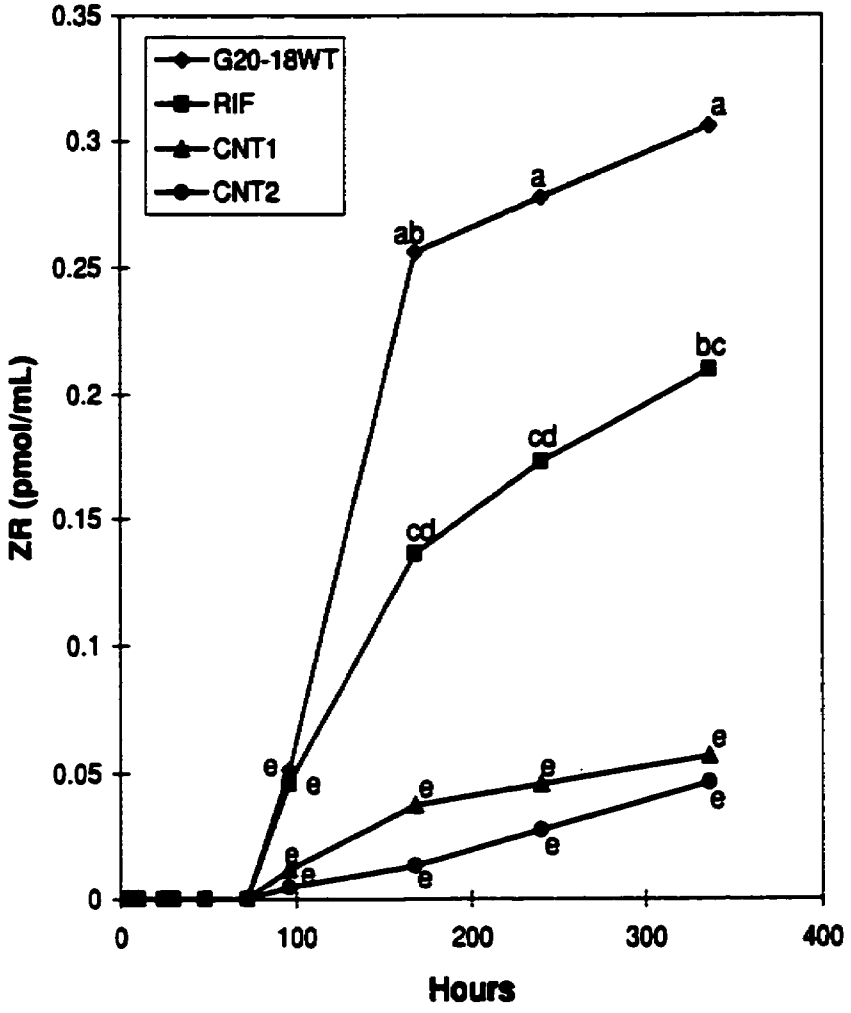


Figure 4.4. Time-course of trans-zeatin Ribose (ZR) by *P. fluorescens* strain G20-18WT and selected mutants growing in pure cultures.
Means followed by the same letter/s are not significantly different as determined by Tukey's test at the rejection level of $P = 0.05$.

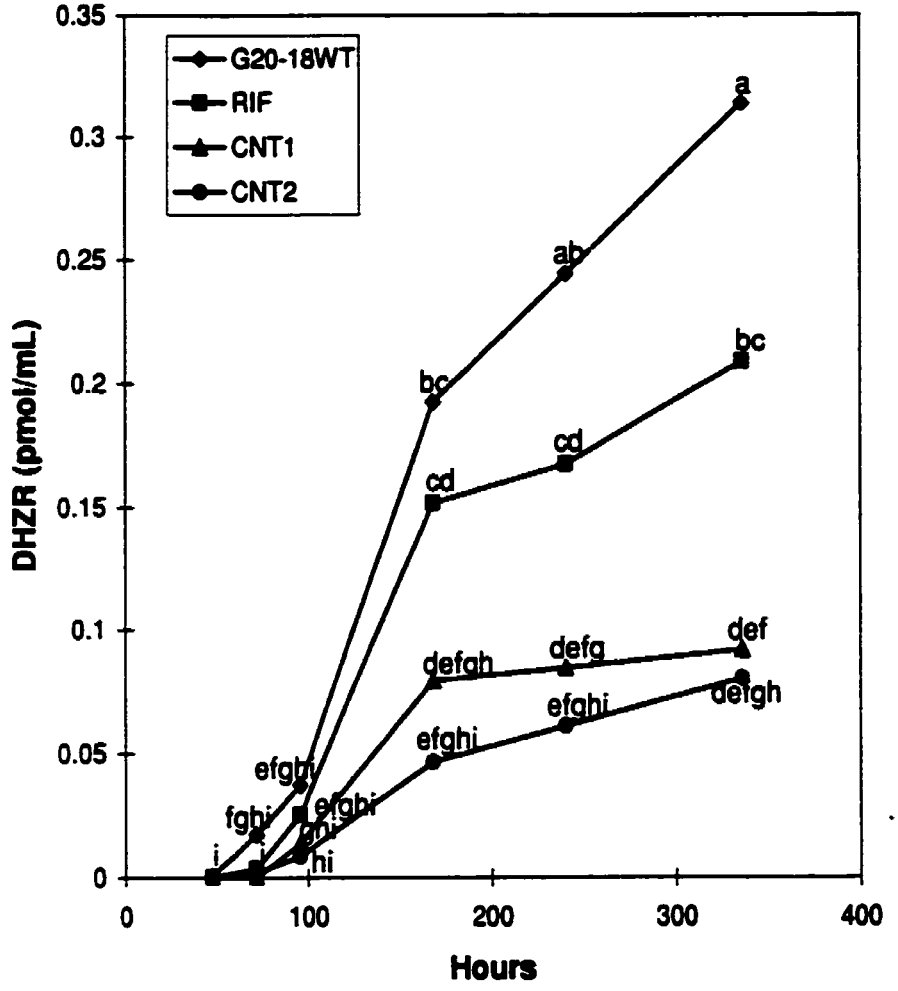


Figure 4.5. Time-course of dihydrozeatin riboside (DHZR) by *P. fluorescens* strain G20-18WT and selected mutants growing in pure cultures.
Means followed by the same letter/s are not significantly different as determined by Tukey's test at the rejection level of P =0.05. Data points, which overlap or are very close are described once with the respective letter/s.

Table 4.3. Percentages of IPA, ZR and DHZR of total [IPA+ZR+DHZR] produced between 96 and 336 h in pure cultures.

	% of [IPA+ZR+DHZR]			
	P. fluorescens strains			
	G20-18WT	RIF	CNT1	CNT2
96 h				
IPA	94.9	95.8	98.3	99.1
ZR	3.0	2.7	0.8	0.3
DHZR	2.2	1.5	0.9	0.6
168 h				
IPA	90.3	90.3	94.4	94.4
ZR	5.6	4.6	1.8	1.3
DHZR	4.2	5.1	3.8	4.3
240 h				
IPA	91.6	90.9	83.2	87.7
ZR	4.5	3.8	2.6	2.8
DHZR	3.9	3.7	4.9	6.1
336 h				
IPA	92.6	93.6	83.1	84.4
ZR	3.7	3.2	6.4	5.7
DHZR	3.8	3.2	10.4	9.9

Because mutants CNT1 and CNT2 were impaired in their ability to produce cytokinins in pure cultures, they were considered useful for testing the formulated hypothesis (page 3). Further studies were done to characterize strain G20-18WT and the selected mutants.

TLC performed with 11 standard cytokinins in solution showed that six cytokinin-separation groups could be distinguished (Table 4.4). Ade could be identified as a separate group, as could IPA/IP, Z/DHZ, ribosides ZR/DHZR,

Table 4.4. Cytokinin separation groups on the basis of their R_F on TLC plates.

Cytokinin-Separation Groups	R_F [1]
Ade	0.58
IPA / IP	0.76
Z / DHZ	0.64
ZR / DHZR	0.54
Z7G / ZOG	0.30
AMP/ ZRNT	<0.05

[1] $R_F = \frac{\text{distance of spot centre from start point}}{\text{distance of solvent front from start point}}$

glucosides Z7G/ZOG and nucleotides AMP/ZRNT. One cytokinin of each separation group was always co-chromatographed with extracts of bacterial cultures and respective spots were checked as described in Table 4.4.

The chromatograms of the *n*-butanol fractions of 72, 96 and 168-h samples of G20-18WT cultures showed the presence of cytokinins belonging to IPA/IP and ZR/DHZR groups and the presence of Z-nucleotide derivatives. No further Z-nucleotide identification was done for these samples. No spots were observed at R_F 0.64 which is the chromatogram location for the Z/DHZ separation group in the TLC system used.

TLC chromatograms of *n*-butanol fractions of 14-d-old cultures of strain G20-18WT and the selected mutants RIF, CNT1 and CNT2 showed clear spots at R_F 0.30, 0.54 and 0.76 indicating the presence of three known cytokinin-separation groups (Table 4.4) in the samples. An unidentified

cytokinin-separation group was observed at R_F 0.45 for strain G20-18WT and mutants RIF and CNT1.

Electrospray HPLC-MS was used to qualitatively confirm the presence of cytokinins in *n*-butanol eluted samples. Analysis of IPA, ZR and DHZR showed the same parent ions and the same diagnostic transitions used in MRM for each cytokinin as listed by Prinsen et al. (1995). Table 4.5 shows the integrated area units of the corresponding peak signals of the analyzed cytokinins. Because internal standards were not used, concentrations could not be determined using this technique. Prinsen et al. (1995) stated that a linear regression function is adequate to describe the relation between integrated area units and concentrations ranging from 1-100 pmol injected. These data indicated that cytokinin concentrations were lower than 0.1 pmol injected. Table 4.5 shows that ZR and DHZR were present in all samples. IPA was present in all 336-h samples. Samples of G20-18WT at 72 h of growth showed an excessive tailing, which is indicative of large concentrations of other compounds. The reduced volume following cleaning of the samples could have contributed to the inability to detect IPA in samples of G20-18WT at 96 and 168 h of growth. Integrated area units were affected by the type of solvent used to elute the spots from the TLC plates. Extracts of 336-h samples of G20-18WT eluted with ethanol:water (50:50 v/v) showed smaller unit areas than the same sample extracts eluted with absolute methanol for all cytokinins.

Table 4.5. Integrated area units of IPA, ZR and DHZR peak signals obtained by electrospray HPLC-MS.

Strains	Culture Age (h)	TLC [1] Elution Solvent	Integrated Area Units		
			IPA	ZR	DHZR
G20-18WT	72	E:W	425730	537	242
G20-18WT	96	E:W	0	61	267
G20-18WT	168	E:W	0	78	272
G20-18WT	336	E:W	54	77	267
G20-18WT	336	Methanol	3263	1120	1642
RIF	336	Methanol	5922	1058	1631
CNT1	336	Methanol	4247	1179	1800
CNT2	336	Methanol	7628	949	1706

[1] TLC performed in solvent n-butanol:water:14N ammonia (60:20:10, v/v) with 20 µL of culture extracts. Cytokinin standards were cochromatographed with the samples. TLC plates were eluted with ethanol:water (E:W) (50:50, v/v) or absolute methanol.

4.1.2.2. Production of IAA and gibberellin-like substances

TLC was performed using chloroform:ethyl acetate:formic acid and showed R_F of 0.12, 0.55 and 0.80 for $GA_{4/7}$, GA_3 and IAA, respectively. A control mixture containing 1 *mM* IAA and 10 *mM* GA_3 showed clear separation of spots at the R_F observed for the pure substances. Chromatograms of ethyl acetate fractions of 72, 96, 168 and 336-h samples of G20-18WT cultures and 336-h samples of selected mutants RIF, CNT1 and CNT2 did not show spots at the R_F corresponding to the standard cochromatographed IAA. However, IAA production could be estimated in sterile supernatants using ELISA test immunoassays but no significant differences were observed among the strains

(Table 4.6). Twenty-µl-samples of ethyl acetate extracts of the control mixture and pure IAA solutions both containing 1 m*M* of IAA placed on TLC plates were calculated to have 3.5 µg of IAA. Maximum possible amounts of IAA in 20-µL-bacterial samples placed on TLC plates as estimated from ELISA immunoassay data were less than 0.026 µg which are probably too low to be detected on the TLC plates (Table 4.6).

Table 4.6. Indole Acetic Acid (IAA) production by *P. fluorescens* strains G20-18WT and selected mutants growing in pure cultures.

Strains	Culture Age [1] (h)	IAA production [2] (nmol ml^{-1})
G20-18WT	72	2.90 (0.15)
G20-18WT	96	3.33 (0.23)
G20-18WT	168	4.62 (0.21)
G20-18WT	336	7.54 (0.48)
RIF	336	6.45 (0.42)
CNT1	336	5.10 (0.46)
CNT2	336	6.64 (0.59)

[1] Strains were cultured in Erlenmeyer side-armed flasks with 100 ml of MM+Gl medium for 14 d.

[2] IAA production was estimated using ELISA test immunoassays. Values are means of three experiments with three replicated flasks. Numbers in parentheses are standard errors of the means.

Chromatograms of ethyl acetate fractions showed that the samples contained gibberellin-like substances. Spots were observed for all samples at R_F ranging between 0.10 and 0.70. Chromatograms were eluted from three R_F sections and a lettuce hypocotyl bioassay was used for activity detection of gibberellin-like substances. R_F sections 0.0-0.2 and 0.4-0.7 contained spots at R_F 0.12 and 0.80,

as described above for authentic gibberellins, $GA_{4/7}$ and GA_3, respectively. Although clear spots at the R_F section 0.2-0.4, observed for certain samples, could not be compared with a standard gibberellin compound, they were eluted and included in the bioassay (Table 4.7). Ethyl acetate eluted fractions of strain

Table 4.7. Effect on lettuce hypocotyl elongation of water and TLC-eluted samples of ethyl acetate fractions of gibberellic acid control solution and *P. fluorescens* strain G20-18WT and selected mutants growing in pure cultures.

PGPR Strains	Period of Growth (h) [1]	Hypocotyl Elongation [2,3] (cm plant^{-1}) R_F		
		0.0-0.2	0.2-0.4	0.4-0.7
G20-18WT	72	0.83 b	0.98 b	0.89 b
G20-18WT	96	0.85 b	0.95 b	1.01 b
G20-18WT	168	0.87 b	0.99 b	1.07 b
G20-18WT	336	0.97 b	1.00 b	1.21 b
RIF	336	0.93 b	1.06 b	1.12 b
CNT1	336	1.31 b	1.17 b	1.07 b
CNT2	336	1.24 b	0.94 b	1.06 b
Water	-	1.03 b		
GA_3 [4]	-			2.63 a

[1] Age of bacterial cultures prior to extraction.

[2] Values are means of three experiments. Means followed by the same letter are not significantly different as determined by Tukey's test at the rejection level P =0.05.

[3] TLC performed in solvent chloroform:ethyl acetate:formic acid (50:20:10, v/v) with 20 µL of culture extracts.

[4] TLC spots of authentic GA_3 present in cochromatographed extracts of control mixture solution containing 10 mM of GA_3 were also eluded and included in the bioassay.

G20-18WT and selected mutants did not differ from the water control in the lettuce-seedling hypocotyl elongation assay (Table 4.7).

The ethyl acetate fraction of the control mixture solution showed a clear spot at R_F 0.55 for GA_3, which produced a significant increase in hypocotyl elongation of lettuce seedlings equal to 2.63 cm pl^{-1} (Table 4.7). This hypocotyl elongation value represents a concentration equal to 1.9×10^4 $\mu g\ L^{-1}$ of GA_3 in the eluted sample, which was estimated from the dose-response curve developed with pure GA_3 solutions (Appendix B).

4.1.2.3. Carbon source utilization

Color development of Biolog plates, expressed as AWCD, followed a sigmoidal curve with incubation time (Fig. 4.6). Color development was not observed for control wells nor during lag phase. No significant differences in AWCD were observed among the strains up to 48 h (P =0.05) (Fig. 4.6). After 72 h of incubation, AWCD of mutant RIF, grown on both TSA or PAF+Rif plates, was different from that of G20-18WT and CNT2 but not from that of CNT1. Overall rate of color development during the linear phase (approximately between 5 and 30 h of incubation) was obtained by dividing the difference of AWCD values by the incubation time. Rate of color development was very similar for all strains and on average equal to 0.015 absorbance units h^{-1}. Biolog plates of strain G20-18WT, mutant RIF grown on TSA and PAF+Rif media, and transconjugants CNT1 and CNT2 had on average 41, 45, 50, 8 and 28 response wells which did not show a qualitative color response, after 72 h of incubation. As stated by Garland (1996), correlation of coordinates of samples for the first principal component (PC) with AWCD values was low enough (r^2=-0.41) to

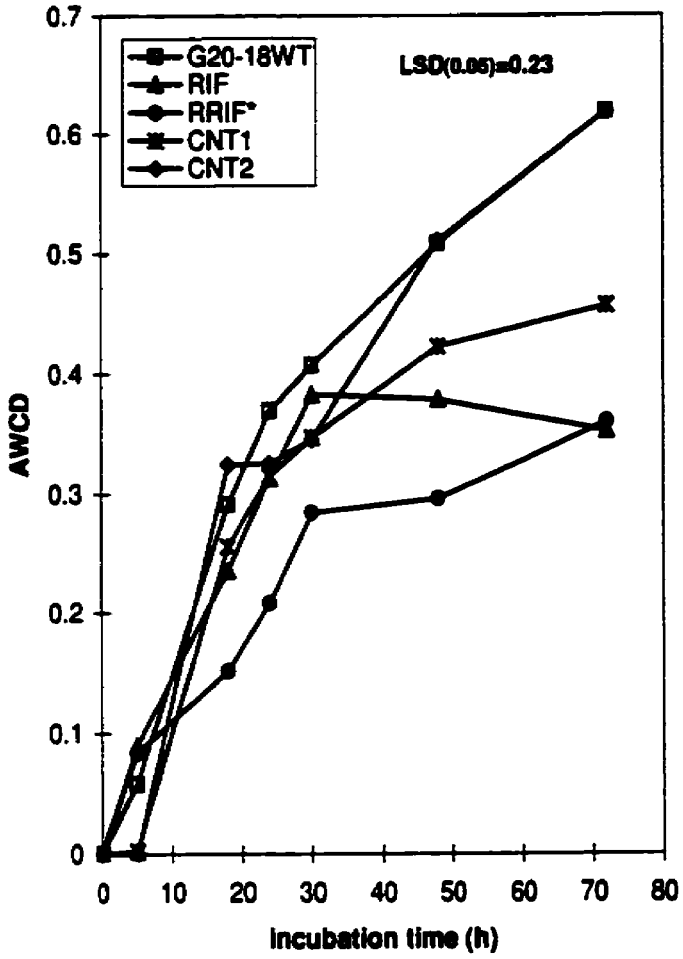


Figure 4.6. Average well color development (AWCD) with incubation of Biolog plates inoculated with *P. fluorescens* strain G20-18WT and selected mutants.

*RRIF indicates that mutant RIF was grown on PAF+Rif plates.

indicate that PCA extracted a pattern in the structure of the data that was not influenced by differences in the rate of color development among plates. Strains showed distinctive patterns of sole-C-source utilization on the basis of PCA of qualitative color response data (Fig. 4.7). Transconjugants CNT1 and CNT2 had much lower coordinate values (PC scores) for the first PC (PC1), which explained 65% of the variance in the data, than either strain G20-18WT or mutant RIF regardless of the media used to grow this strain. However, incubation times had an effect on the PC scores assigned to the different strains. All strains had similar PC1 scores at 5 h of incubation. However, mutants RIF, CNT1 and CNT2 had significantly lower PC scores than strain G20-18WT for the second PC (PC2), which explained 12% of the variance in the data (P =0.01). At 18 and 24 h of incubation, mutant RIF showed significantly lower PC2 scores than strain G20-18WT, but after 30 h of incubation both strains had values for PC2 which were too similar to be separated (P =0.01). Transconjugants CNT1 and CNT2 had similar values for PC1, but had different PC2 scores with coordinate values varying with the incubation time. Transconjugant CNT1 always had significantly higher PC2 scores than transconjugant CNT2 for each incubation time (P=0.01). Significant differences were observed among PC2 scores of the different incubation times (P=0.01). Transconjugant plates incubated for 18 h had lower PC2 scores than PC2 scores of other incubation times that followed a chronological order.

The separation of the strains in PC space can be related to differences in C-source utilization by examining the correlation of the original variables to the

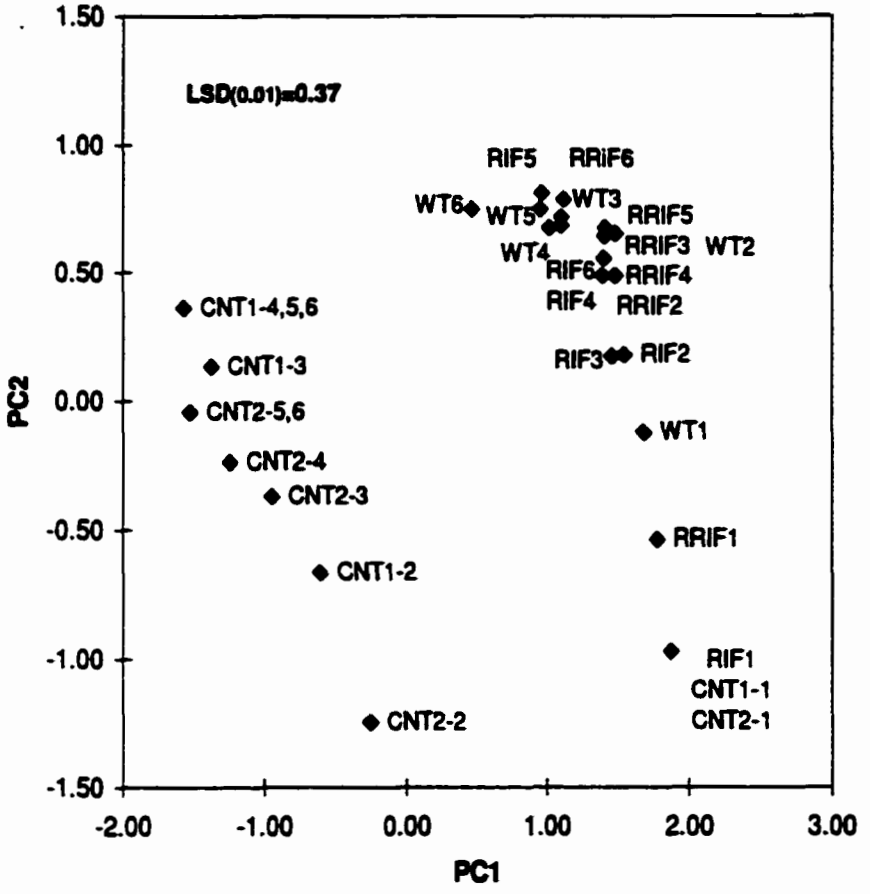


Figure 4.7. Principal component analysis (PC) of *P. fluorescens* strain G20-18WT and mutants RIF, CNT1 and CNT2.

Names of strains, as described in Fig. 4.6, followed by 1, 2, 3, 4, 5 and 6 indicate 5, 18, 24, 30, 48 and 72 h of incubation of the Biolog plates, respectively. Points labeled with more than one name or incubation time indicate the same coordinates values. LSD(0.01), Least significant difference at the rejection level P =0.01 for both PC1 and PC2 scores.

PCs. C-sources which had at least 60% of their variance explained by PC1 or PC2 (57 out of 95 variables in the Biolog plate) were considered relevant to differentiate among PGPR strains (Table 4.8). Poor correlation of a C source to a PC does not necessarily mean that it was poorly utilized by the strains, but rather that its utilization was not different among strains and that it could not help to differentiate them. Analysis of PC1 (Fig. 4.7) indicated that strain G20-18WT and mutant RIF utilized the C sources that correlated to PC1 (Table 4.8) to a relatively greater degree than transconjugants CNT1 and CNT2. On the basis of PC2 analysis, effects of strains and incubation times could be separated. Thus, PC2 distinguished the strain G20-18WT from all the mutants at 5 h of incubation (Fig. 4.7). Strain G20-18WT showed greater relative response in wells containing D-galactose, α-D-glucose, D-mannose, p-hydroxy-phenylacetic acid, glycogen and thymidine than the mutants (Table 4.8). PC2 also separated plates of mutant RIF from plates of strain G20-18WT incubated for 18 and 24 h (Fig. 4.7). Transconjugant CNT2 utilized the C-sources that were negatively correlated to PC2 to a relatively greater degree than transconjugant CNT1 (Fig. 4.7 and Table 4.8).

4.1.3. Effects of Adenine (Ade) Addition on Cytokinin Production by *P. fluorescens* Strain G20-18WT.

Fig. 4.8 shows the effects of Ade on the production of [IPA+ZR+DHZR] by *P. fluorescens* strain G20-18 WT growing in MM+Gl medium for 96 and 168 h. This strain produced the highest amounts of total cytokinins with Ade additions of 10^{-7} M and 10^{-5} *M* after 96 and 168 h of growth, respectively.

Table 4.8. Correlation of C-sources with the two principal components determined in the analysis of Biolog plates for *P. fluorescens* strain G20-18WT and selected mutants.

PC1		PC2	
C-source	r^2 [1]	C-source	r^2
Carbohydrates		Carbohydrates	
N-Acetyl-D-glucosamine	-0.94	D-Fructose	-0.72
D-Arabitol	-0.84	D-Galactose	0.76
Mono-methylsuccinate	-0.96	Gentiobiose	-0.75
Carboxylic acids		α-D-Glucose	0.74
cis-Aconitic acid	0.91	Maltose	-0.73
Citric acid	0.96	D-Mannitol	-0.76
Formic acid	0.98	D-Mannose	0.74
D-Galacturonic acid lactone	0.98	D-Psicose	-0.61
D-Galacturonic acid	0.74	Sucrose	-0.73
D-Glucosaminic acid	0.75	D-Trehalose	-0.72
D-Glucuronic acid	0.81	Turanose	-0.72
Itaconic acid	-0.81	Carboxylic acids	
α-keto-Butyric acid	-0.77	p-Hydroxy-phenylacetic acid	0.78
α-keto-Glutaric acid	-0.93	Acetic acid	-0.68
α-keto-Valeric acid	-0.89	D-Gluconic acid	-0.68
Malonic acid	-0.82	β-Hydroxybutyric acid	-0.68
Quinic acid	-0.94	Polymers	
D-Saccharic acid	-0.96	Glycogen	0.82
Sebacic acid	-0.69	Dextrin	-0.73
Amino acids		Tween 40	-0.74
Glycyl-L-aspartic acid	-0.81	Aromatic Chemicals	
Glycyl-L-glutamic acid	-0.93	Thymidine	0.90
D-Alanine	-0.74		
L-Aspartic acid	-0.77		
L-Leucine	-0.92		
L-Ornithine	-0.87		
L-Proline	-0.93		
L-Pyroglutamic acid	-0.90		
D-Serine	-0.81		
L-Serine	-0.93		
L-Threonine	-0.86		
D,L-Carnitine	-0.93		
γ-AminoButyric acid	-0.93		
Amides			
Succinamic acid	-0.82		
Glucuronamide	-0.81		
Alaninamide	-0.82		
Amines			
Putrescine	-0.96		
2-Amino ethanol	-0.84		
Aromatic Chemicals			
Urocanic acid	-0.93		
Inosine	-0.96		

[1] r^2, Pearson's correlation coefficient.

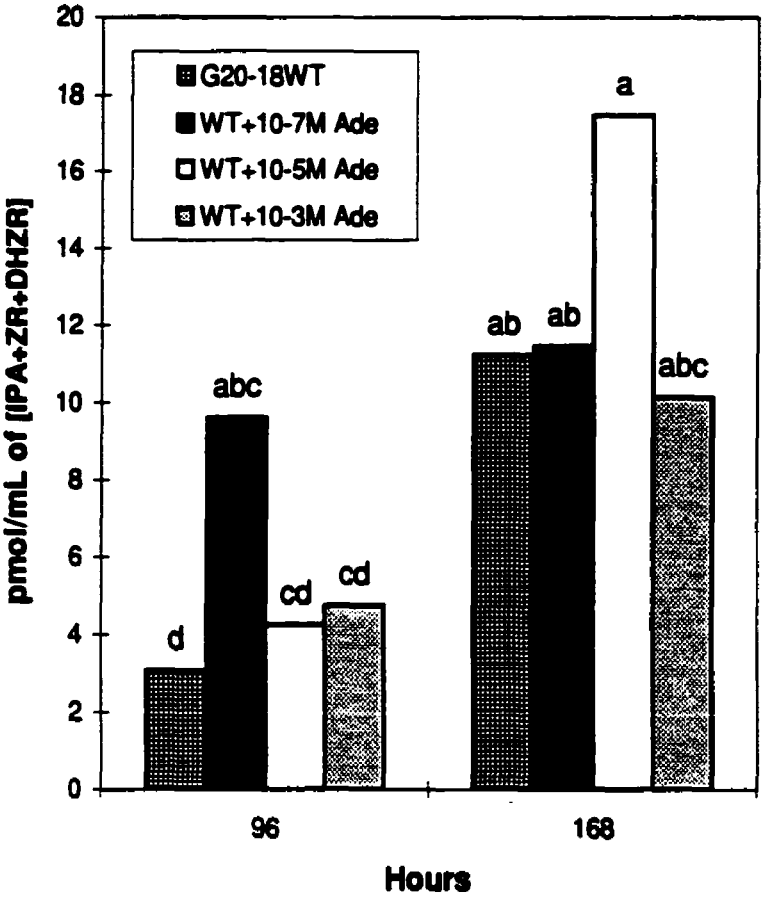


Figure 4.8. Effect of adenine (Ade) on cytokinin production [IPA+ZR+DHZR] by *P. fluorescens* strain G20-18WT grown in MM+Gl medium.

Bars are means of three experiments with three replicated flasks. Means with the same letter/s are similar as determined by Tukey's test (P =0.05).

However no significant differences were observed among treatments after 168 h. Addition of 10^{-7} *M* Ade significantly increased cytokinin production at 96 h of growth by 68 % with respect to the control (P =0.05).

Addition of Ade had different effects on the production of IPA, ZR and DHZR. Fig. 4.9 shows that the effects of Ade on IPA production were similar to those observed in Fig. 4.8 because IPA production after 96 and 168 h of growth represented 98.5 and 97.5%, respectively, of the total amount of [IPA+ZR+DHZR] estimated using immunoassays. IPA production after 96 and 168 h of growth was the greatest at 10^{-7} *M* and 10^{-5} *M* Ade with 9.54 and 17.06 pmol mL^{-1} IPA and these were greater than the controls by 68% and 35%, respectively. However no significant differences were observed among treatments after 168 h. The production of ZR and DHZR was 100-fold lower than IPA for G20-18WT. The effects of Ade additions on ZR and DHZR production are shown in Figs. 4.10 and 4.11. No differences among treatments were observed in the amounts of ZR estimated in 96-h-culture filtrates. Only addition of 10^{-3} *M* Ade significantly increased ZR production by 39% at 168 h (P=0.05) (Fig. 4.10). DHZR production by G20-18WT after 96 h was as low as 0.0147 pmol mL^{-1} but addition of 10^{-5} *M* Ade significantly increased DHZR production 11 fold at 96 h (P =0.05) (Fig. 4.11). Amounts of DHZR in 168-h-old sterile supernatants of G20-18WT were 0.14 pmol mL^{-1}. DHZR production was significantly increased 2.4 and 2.3 fold with the addition of 10^{-5} and 10^{-3} *M* Ade (Fig. 4.11).

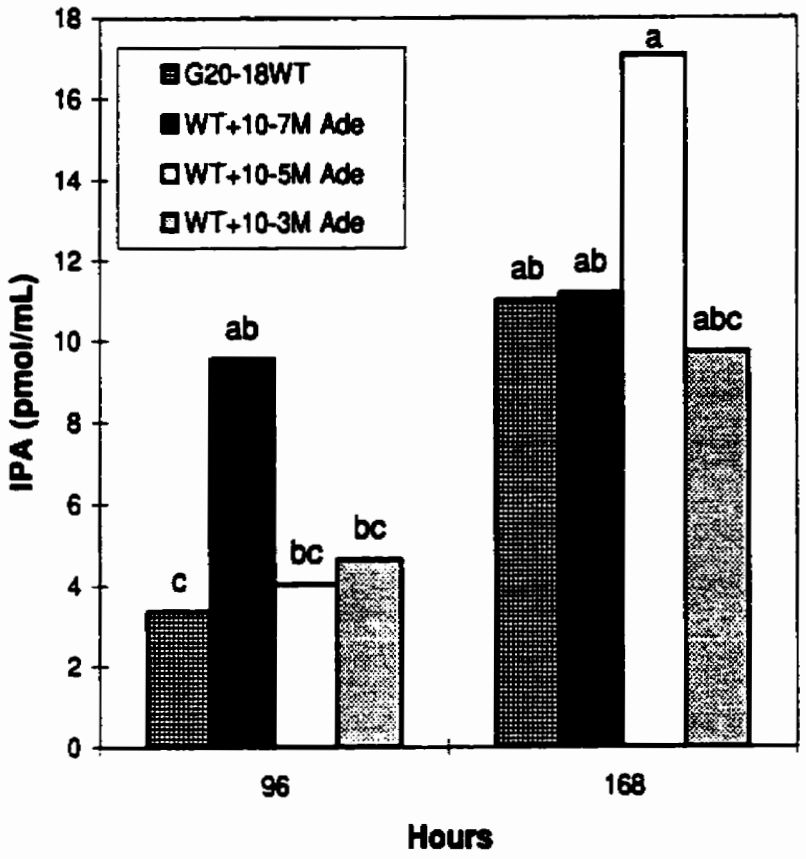


Figure 4.9. Effect of adenine (Ade) on the production of isopentenyl adenosine (IPA) by *P. fluorescens* strain G20-18WT grown in MM+Gl medium.

Bars are means of three experiments with three replicated flasks. Means with the same letter/s are similar as determined by Tukey's test (P =0.05).

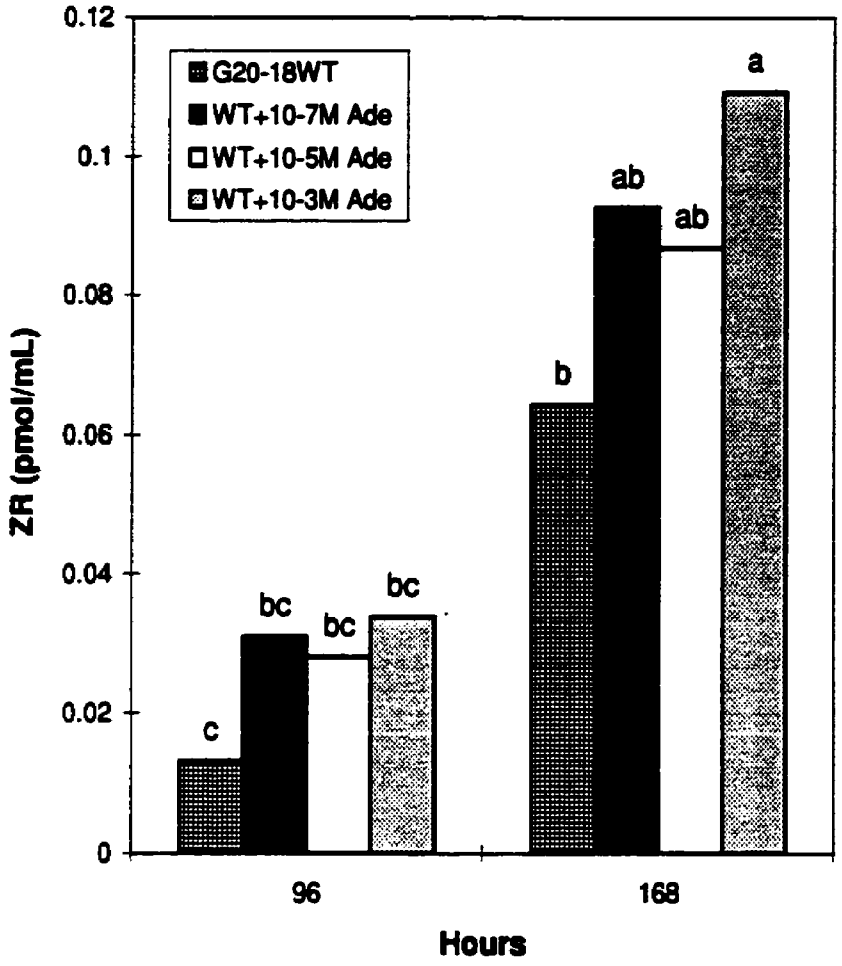


Figure 4.10. Effect of adenine (Ade) on the production of zeatin riboside (ZR) by *P. fluorescens* strain G20-18WT grown in MM+Gl medium.
Bars are means of three experiments with three replicated flasks. Means with the same letter/s are similar as determined by Tukey's test (P=0.05).

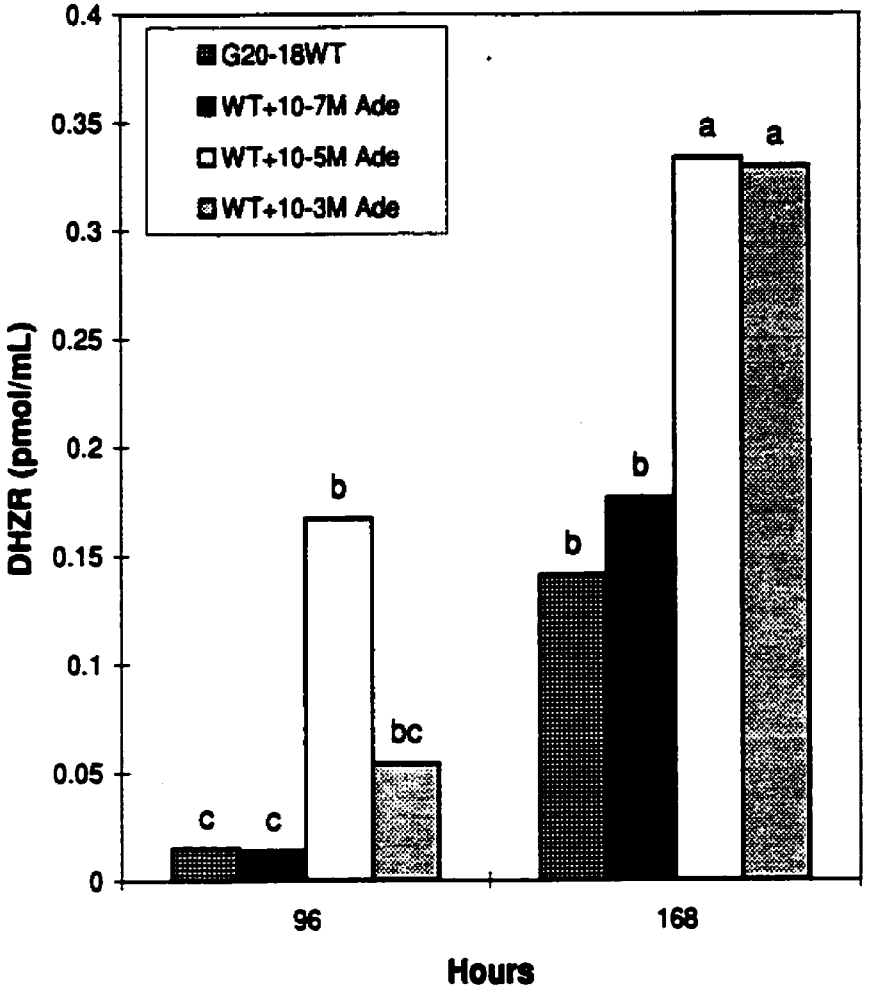


Figure 4.11. Effect of adenine (Ade) on the production of dihydrozeatin zeatin riboside (DHZR) by *P. fluorescens* strain G20-18WT grown in MM+Gl medium.

Bars are means of three experiments with three replicated flasks. Means with the same letter/s are similar as determined by Tukey's test (P =0.05).

Growth curves of the strains in MM+Gl medium with varying concentrations of Ade were very similar to those depicted in Fig. 4.2 and showed no differences among treatments (data not shown). In contrast to the results for cytokinin production, no differences were observed in bacterial numbers in samples taken in stationary phase, after 96 and 168 h (data not shown).

Thus, the production of IPA, ZR and DHZR by G20-18WT could be increased by addition of the precursor Ade. However the type of response varied among the three cytokinins assayed.

4.1.4. Effects of Wheat Exudate on the Growth of *P. fluorescens* Strain G20-18WT.

Fig. 4.12 shows the effects of wheat exudate added into MM+Gl medium on the growth of *P. fluorescens* strain G20-18WT. No differences were observed among treatments between 4 and 10 h. Addition of 20 ml L^{-1} of wheat exudate produced significantly lower bacterial numbers than controls and other doses of wheat exudate between 24 and 168 h (P=0.05).

4.2. *Pseudomonas* PGPR in Association with Whole Plant Systems

4.2.1.*Triticum aestivum* (Wheat) cv. Katepwa

4.2.1.1. Growth pouches (GP)

The effects of *P. fluorescens* strain G20-18WT, mutant RIF and five selected transconjugants on the initial growth of Katepwa wheat are depicted in

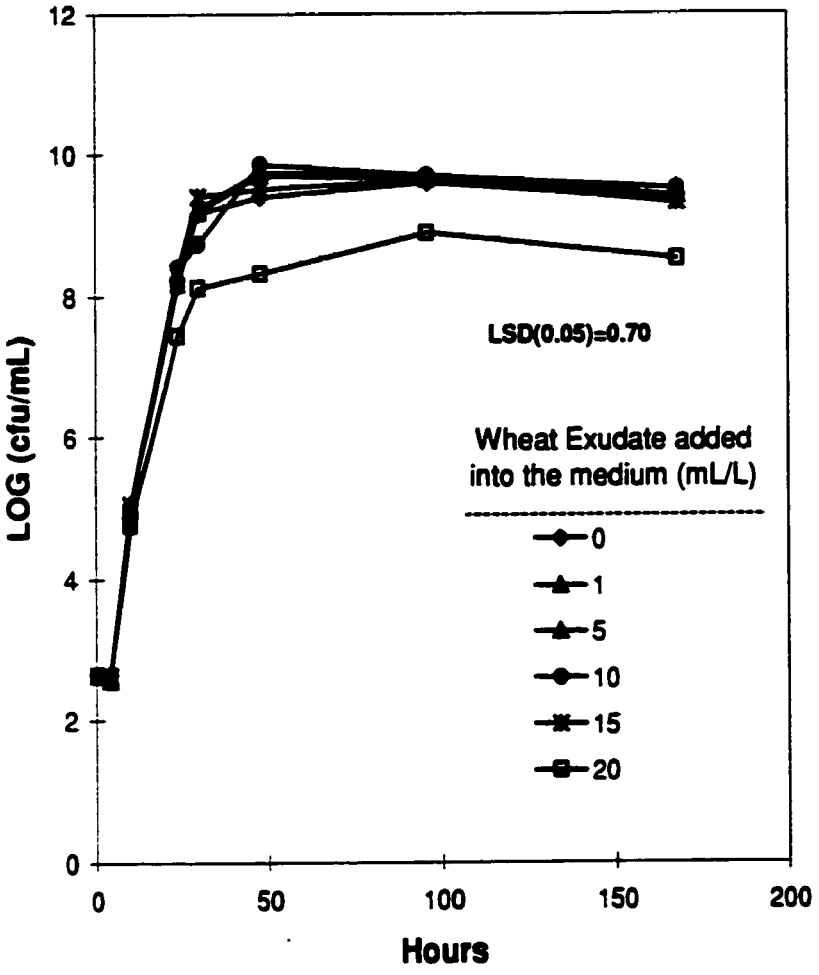


Figure 4.12. Effect of Wheat Exudate on the Growth of *P. fluorescens* strain G20-18WT grown in MM+Gl medium.
Values are means of three experiments with three replicated flasks.
LSD(0.05): Least significant difference at the rejection level P =0.05.

Table 4.9. The percent germination was not significantly modified by the inoculation treatments (Table 4.9).

Table 4.9. Effect of *P. fluorescens* strain G20-18WT, mutant RIF and five transconjugants on germination, root and shoot length and root colonization of Katepwa wheat seedlings grown in growth pouches.

Inoculation Treatments	Germination (%) [1,2]	Length [1] (cm plant^{-1})		Log [1,3] (cfu g^{-1} of root dry weight)
		Root	Shoot	
Control	80.4 ± 0.8	16.2 ± 1.1	9.6 ± 0.6	n.d. [1]
G20-18WT	86.9 ± 0.6	18.1 ± 0.7	10.6 ± 0.7	7.5 ± 0.0
RIF	86.9 ± 0.7	17.6 ± 1.0	10.0 ± 0.9	7.4 ± 0.1
Tn4 (CNT1)	81.1 ± 1.0	16.1 ± 0.7	9.0 ± 0.8	7.4 ± 0.1
Tn7	85.7 ± 0.7	16.5 ± 0.4	9.7 ± 0.3	7.0 ± 0.0
Tn8	81.7 ± 0.7	17.1 ± 0.8	8.9 ± 0.4	7.5 ± 0.1
Tn10	83.3 ± 0.9	16.9 ± 1.3	10.0 ± 0.2	7.2 ± 0.1
Tn11 (CNT2)	83.3 ± 0.8	16.4 ± 0.5	8.5 ± 0.2	7.3 ± 0.1

[1] Means and standard errors. Seedlings were harvested 7 d after planting. Data are means of three experiments.
[2] Percent of germination was calculated for each GP.
[3] Counts of G20-18WT, RIF, and transconjugants were performed on PAF, PAF+Rif and PKS plates, respectively. n.d. not detectable.

Seeds soaked in bacterial suspensions containing 10^9cfu mL^{-1} for 3 h had a bacterial density higher than 3 x 10^4 cfu seed^{-1}. Root colonization of Katepwa wheat seedlings grown for 7 d in GP did not show significant differences between *P. fluorescens* strain G20-18WT and the selected mutants (Table 4.9).

4.2.1.2. Greenhouse

Table 4.10 shows the effect of *P. fluorescens* strain G20-18WT and selected mutants on the emergence of Katepwa wheat under greenhouse conditions at 4, 10 and 17 d after planting (d.a.p.). Emergence was significantly increased with

strain G20-18WT and mutant RIF (P=0.05). Seeds inoculated with transconjugants Tn4 and Tn11 reached their maximum emergence at 4 d.a.p. and emergence at 10 and 17 d.a.p. was significantly (P=0.05) lower than that for non-inoculated seeds. Emergence of seeds inoculated with strains G20-18WT and RIF significantly increased up to 69 and 66 % at 10 d.a.p, respectively and continued increasing up to 76% for both strains at 17 d.a.p (P=0.05).

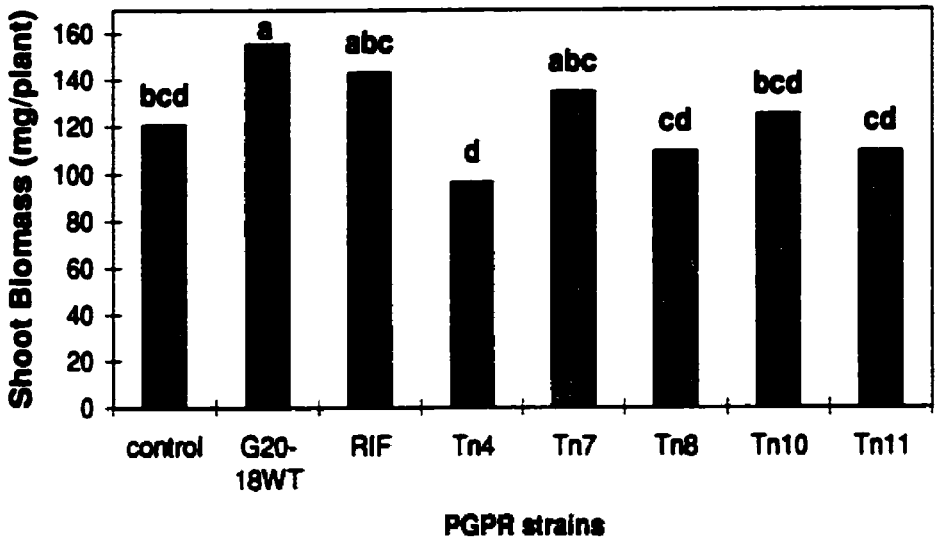
Table 4.10. Effect of *P. fluorescens* strain G20-18 WT and selected mutants on the emergence of Katepwa wheat under greenhouse conditions.

	% Emergence at days after planting [1]		
	4	10	17
Control	21	34	38
G20-18WT	48	69	76
RIF	48	66	76
Tn4	10	10	10
Tn7	10	34	35
Tn8	10	28	31
Tn10	10	31	31
Tn11	10	10	10
LSD $P = 0.05$	12	13	11

[1] Means of three replicates. Each replicate consisted of 96 seeds per tray with 48 pots planted with two seeds.

Fig. 4.13 shows the effect of *P. fluorescens* strain G20-18WT, mutant RIF and five transconjugants on shoot and root growth of Katepwa wheat at 21 d.a.p.

A.



B.

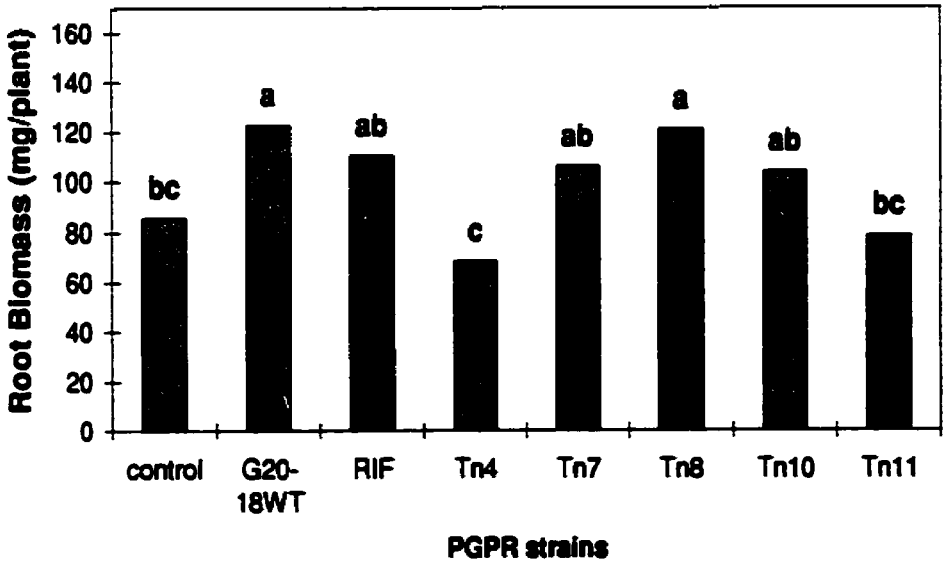


Figure 4.13. Effect of *P. fluorescens* strain G20-18WT, mutant RIF and five transconjugants on shoot and root growth of Katepwa wheat under greenhouse conditions.

Bars are means of three replicates. Each replicate was a tray with 48 pots. Bars with the same letter/s are not different as determined by Tukey's test at the rejection level $P = 0.05$.

Inoculation with strain G20-18WT significantly increased shoot biomass by 22% (P=0.05). Transconjugants Tn4, Tn8, Tn10 and Tn11 produced lower shoot biomass than strain G20-18WT whose value was 154.9 mg plant^{-1} (Fig. 4.13A). Inoculation with strain G20-18WT and transconjugant Tn8 significantly increased root biomass by 30% relative to the control (Fig. 4.13B).

There were no differences in root and shoot colonization of Katepwa wheat by *P. fluorescens* strain G20-18WT, mutant RIF and five transconjugants after 21 d under greenhouse conditions (Table 4.11). No bacteria were detectable

Table 4.11. Root and shoot colonization of Katepwa wheat by *P. fluorescens* strain G20-18WT, mutant RIF and five transconjugants under greenhouse conditions.

Strains	LOG [cfu / g dry weight] [1]			
	Root		Shoot	
	PAF	Selective Media [2]	PAF	Selective Media
Control	nd [3]	nd	nd	nd
G20-18WT	7.4 ± 0.1	nd	6.8 ± 0.1	nd
RIF	7.7 ± 0.1	5.8 ± 0.1	6.6 ± 0.2	4.2 ± 0.3
Tn4	7.0 ± 0.4	5.9 ± 0.1	7.1 ± 0.2	5.5 ± 0.3
Tn7	7.0 ± 0.1	5.6 ± 0.1	6.8 ± 0.1	4.5 ± 0.5
Tn8	7.5 ± 0.1	5.8 ± 0.1	6.9 ± 0.1	4.8 ± 0.4
Tn10	7.3 ± 0.1	4.9 ± 0.1	6.8 ± 0.1	4.4 ± 0.2
Tn11	7.0 ± 0.5	5.7 ± 0.1	6.9 ± 0.1	5.7 ± 0.3
LSD (P = 0.05)	2.0	1.4	0.8	1.6

[1] Mean and standard error of three replicates plated on PAF and respective selective media.
[2] Selective media, PAF + 100 µg mL^{-1} of Rifampicin for RIF mutant and PKS for transconjugants.
[3] nd, not detectable.

from non-inoculated seedlings. Samples of non-inoculated plants and plants inoculated with G20-18WT were also plated on selective media, but bacteria were not detectable.

4.2.1.3. Growth Chamber

The emergence of Katepwa wheat seedlings at 8 d.a.p was significantly increased by 14 and 13% when seeds were inoculated with *P. fluorescens* strain G20-18WT and RIF mutant, respectively (P=0.01) (Table 4.12).

Table 4.12. Effect of *P. fluorescens* strain G20-18WT and mutant RIF on the Emergence of Katepwa wheat grown in a growth chamber.

Inoculation Treatments	% Emergence at 8 days after planting[1]
Control	49 ± 15
G20-18WT	63 ± 11
RIF	62 ± 15
$LSD_{P=0.01}$	10

[1] Means and standard deviations of two experiments with 10 replicated pots.

Although the Redi-earth was autoclaved, the bacterial population of Redi-earth, estimated after sterilization averaged Log 8.1 [cfu g^{-1}] (Table 4.13). Similar bacterial populations, ranging between Log 7.8 and 8.8, were estimated when root samples of Katepwa wheat plants were plated on PAF medium regardless of the inoculation treatments (Table 4.13). Shoots of Katepwa wheat plants had

significantly lower numbers of bacteria than roots at 25 d.a.p. (P=0.05). No bacteria were detected from uninoculated plants when both root and shoot samples were plated on PAF+rif medium. Root and shoot colonization of Katepwa wheat by strain RIF increased 1.4 and 1.3 fold from 8 to 25 d.a.p, respectively.

Table 4.13. Bacterial colonization of roots and shoots of Katepwa wheat *P. fluorescens* strain RIF and native Redi-earth bacteria in a growth chamber.

	LOG [cfu g^{-1} root dry weight] at days after planting [1]					
	8		15		25	
	PAF	PAF+Rif [2]	PAF	PAF+Rif	PAF	PAF+Rif
Control						
Root	7.9	n.d. [3]	7.8	n.d.	8.7	n.d.
Shoot	7.3	n.d.	7.4	n.d.	7.4	n.d
Strain RIF						
Root	7.9	5.5	7.8	6.7	8.8	7.6
Shoot	7.2	3.4	7.1	3.9	7.0	4.3
LSD $_{P=0.05}$	0.8		0.8		0.8	

[1] Means of two experiments with five replicated pots. Samples were plated on PAF and PAF+Rif media.
[2] Both media contained 50 µg mL^{-1} of both cyclohexamide and benomyl.
[3] n.d., not detectable.

Strain G20-18WT had no effect on the number of leaves and the height of Katepwa wheat plants measured at 18, 27, 36 and 46 d.a.p (Table 4.14).

Table 4.14. Effect of *P. fluorescens* strain G20-18WT on number of leaves and height of the Katepwa wheat grown in a growth chamber.

	Leaves [1] (number plant^{-1})		Height [1] (cm plant^{-1})	
D.A.P.	Control	G20-18WT	Control	G20-18WT
18	1.7 ± 0.5	1.7 ± 0.5	21.9 ± 3.8	22.5 ± 3.3
27	3.2 ± 0.5	3.2 ± 0.5	36.1 ± 3.8	35.9 ± 3.1
36	4.6 ± 0.5	4.9 ± 0.3	49.2 ± 3.6	46.1 ± 5.0
46	6.4 ± 0.5	6.6 ± 0.7	57.8 ± 4.0	58.7 ± 3.6

D.A.P.: Days after planting.
[1] Means and standard deviations of two experiments with 30 pots and two plants per pot.

Fig. 4.14. shows the effect of strain G20-18WT on the number of tillers and visible ears of Katepwa wheat. The number of tillers per plant increased from 25 d.a.p. until anthesis (70 d.a.p) ranging between 1.0 and 7.7, respectively.

At 62 and 70 d.a.p., numbers of tillers per plant inoculated with strain G20-18WT were significantly higher than those of uninoculated plants (Fig. 4.14). At anthesis, Katepwa wheat plants inoculated with strain G20-18WT had 4.7 visible ears per plant, which was significantly higher (15%) than the control (P=0.05), (Fig. 4.14).

Total leaf areas of Katepwa wheat plants inoculated with strain G20-18WT were significantly higher than those of uninoculated plants at both 70 and 90 d.a.p, respectively (P=0.05) (Table 4.15). Root biomass of inoculated plants was significantly (P=0.05) higher than root biomass of control plants at 90 d.a.p.

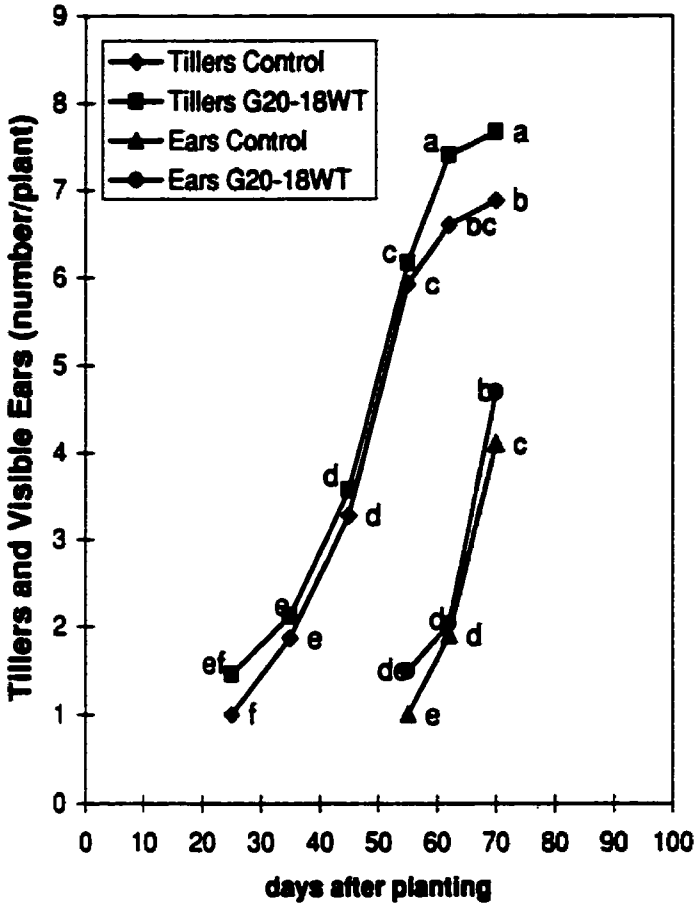


Figure 4.14. Effect of *P. fluorescens* strain G20-18WT on the number of tillers and visible ears of Katepwa wheat grown in a growth chamber.
Means of 60 replicated pots with two plants per pot. Measurement done at 70 d after planting corresponds to anthesis. Tillers represent total numbers per plant. The number of visible ears represents ears that were completely over the canopy. Data points followed by the same letters indicate no statistical differences between means of tillers or ears, as determined by Tukey's test at the rejection level P =0.05.

(P=0.05). The reproductive partition index did not differ between treated and control plants (Table 4.15).

Table 4.15. Effect of *P. fluorescens* strain G20-18WT on total leaf area, biomass of ears, shoots and roots and partition index of Katepwa wheat in a growth chamber.

	Total Leaf Area [2] at days after planting (cm^2 $plant^{-1}$) [1]		Biomass at 20 days after Anthesis (g $plant^{-1}$) [1]			Reproductive Partition Index[4]
	70[3]	90	Ears	Shoot	Root	(%)
Control	78	19	3.2	6.3	4.5	23
G20-18WT	86	33	3.5	6.6	5.2	23
LSD	7	5	0.3	0.3	0.5	2
P =	0.05	0.05	0.10	0.10	0.05	0.10

[1] Means of two experiments with 10 replicated pots and two plants per pot.
[2] Leaf Area was estimated from maximum length and width of every green leaf using the equation described by Miralles and Slafer (1991).
[3] Anthesis was at 70 d.a.p.
[4] Reproductive partition index calculated as the biomass ratio [Ears/(Ears+Shoots+Roots)] x 100.

Yield components, shoot biomass and harvest index of Katepwa wheat grown in a chamber and harvested at 10 d after physiological maturity did not show any significant effect of inoculation with *P. fluorescens* strain G20-18WT (Table 4.16).

Table 4.16. Effect of *P. fluorescens* strain G20-18WT on yield components, shoot biomass and harvest index of Katepwa wheat grown in a growth chamber [1].

	Yield Components				Shoot Biomass	Harvest Index
	Ears		Grains			
	No[2]	g [2]	No	g	g	(%)
Control	4.1±0.5	5.3±0.6	118±12	3.9±0.5	8.6±1.8	28±4
G20-18WT	4.3±0.6	5.5±0.5	120±13	4.1±0.5	8.8±1.6	28±5

[1] Means and standard deviations of two experiments with 20 replicated pots and two plants per pot. Plants were harvested 10 d after physiological maturity that was reached at approximately 110 d.a.p.
[2] Number and grams per plant.

4.2.2. *Raphanus sativus* (Radish) cv. Cherry Belle

4.2.2.1. Growth pouches (GP)

4.2.2.1.1. Screening of PGPR strains

The percent germination of radish seeds inoculated with strains G20-18WT and 63-28 was significantly higher than that of seeds inoculated with strain Ral-3 (Table 4.17) (P=0.05). No significant effects of PGPR strains on root and shoot length of radish cv. Cherry Belle were observed when seedlings were grown for 10 d in GP (Table 4.17) (P=0.05). Dry weight of seedlings inoculated with strains G20-18WT, G8-32 and 63-28, significantly increased by 31, 21 and 19 %, respectively compared to the control (P=0.05).

Table 4.17. Effects of PGPR strains on germination, root and shoot length and dry weight of radish cv. Cherry Belle grown in growth pouches.

PGPR strains	Germination[1] (%)	Length[2] (cm plant^{-1})		Dry Weight[2] (mg plant^{-1})
		Root	Shoot	
Control	93.3±1.2	13.2±0.3	6.0±0.0	5.2±0.2
G20-18WT	95.8±1.4	14.5±0.1	6.8±0.1	6.8±0.2
G8-32	92.5±1.3	14.5±0.2	6.5±0.0	6.3±0.1
GR12-2	93.3±0.9	13.3±0.1	6.5±0.1	5.0±0.2
63-28	95.8±0.7	14.7±0.4	6.9±0.1	6.2±0.3
Ral-3	85.8±1.5	11.0±0.5	5.8±0.0	5.1±0.1
LSD $_{(P=0.05)}$	10.0	ns[3]	ns	0.8

[1] Means and standard errors. Means of three experiments with 10 replicate GP with five seeds. Percent germination was calculated for each GP.
[2] Seedlings were harvested 10 d.a.p. Means and standard errors of three experiments with seven replicate GP.
[3] ns: not significant

Radish seeds inoculated with strain 63-28 had the highest bacterial numbers, which were significantly higher than those counted on seeds inoculated with the other strains (P=0.05) (Fig. 4.15). Bacterial numbers on roots of seedlings grown for 10 d. in GP were similar to those estimated on seeds but, in this case, the differences between strains G8-32 and 63-28 were not significant.

4.2.2.1.2. Dose-response of exogenously-applied cytokinins

4.2.2.1.2.1. *Trans*- zeatin

Whole seedlings of radish cv. Cherry Belle responded to exogenous application of cytokinin Z (Table 4.18). Root length of radish seedlings was significantly increased with the addition of concentrations of Z ranging between 5 and 50 nM (P=0.05). Radish seedlings supplied with 100 nM Z had a similar

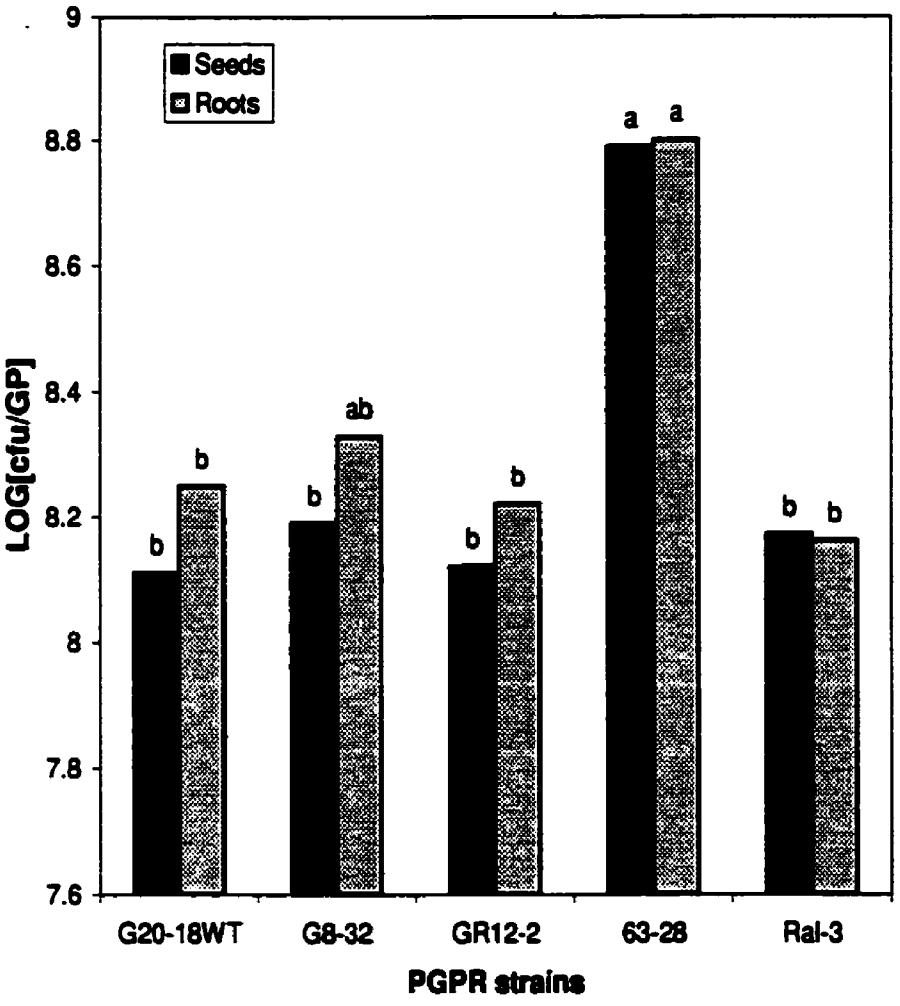


Figure 4.15. Bacterial numbers on seeds and roots of radish cv. Cherry Belle inoculated with PGPR strains and grown in growth pouches.
Bars are means of three experiments with three replicate GP. Bars with the same letters are not different as determined by Tukey's test at the rejection level P=0.05. No bacteria were detected from non-inoculated seedlings. Seeds and Roots represent bacterial numbers on inoculated seeds before planting and roots of 10 d-old seedlings per GP, respectively.

root length to control seedlings. Only shoot length and dry weight of radish seedlings supplied with 50 n*M* Z were significantly different from the control (P=0.05).

Table 4.18. Response of radish cv. Cherry Belle grown in growth pouches to exogenous supply of trans-zeatin.

Z[(1)] concentrations (n*M*)	Length (cm plant^{-1})		Dry Weight (mg plant^{-1})
	Root	Shoot	
0	14.3 c	6.2 b	6.0 b
0.5	14.5 bc	6.4 ab	6.3 ab
5	17.0 ab	6.5 ab	6.6 ab
10	16.8 ab	6.6 ab	7.0 ab
50	17.3 a	7.0 a	7.2 a
100	16.0 bc	6.8 ab	6.7 ab

(1) Concentration of Z in the solution added to GP. GP were filled with 15 mL of Z or $MgSO_4$ solutions. Seedlings were harvested 10 d.a.p.
Data are means of three experiments with 10 replicate GP. Means followed by the same letters are similar as determined by the Tukey pairwise comparison at the rejection level P=0.05.

Germination of radish seeds was not affected by the addition of Z or inoculation with selected PGPR strains (Table 4.19). However, mean percentage germination of inoculated seeds was 95.8% while the mean percentage germination of seeds supplied with Z was 89.5%. Both PGPR inoculation and application of Z significantly increased growth of whole radish seedlings (P=0.05). Z concentrations ranging between 0.5 and 10 n*M* had significant effects on root and shoot length of seedlings but no differences among

concentrations were observed in this range (P=0.05). Dry weight of radish plants increased at all concentrations of Z but the maximum value was observed when plants were supplied with 10 n*M* Z. Radish seedlings inoculated with PGPR strains G20-18WT and G8-32 had similar effects on root and shoot length as concentrations of Z ranging between 0.5 and 10 n*M*. Dry weight of radish seedlings was significantly increased by all PGPR strains and the effects were similar to those obtained by addition of Z at concentrations ranging between 5-100 n*M* (P=0.05).

Table 4.19. Response of radish cv. Cherry Belle grown in growth pouches to exogenous supply of Z or inoculation with PGPR.

Treatments	Germination (%) [1]	Root Length (cm plant^{-1})	Shoot Length (cm plant^{-1})	Dry Weight (mg plant^{-1})
Z (n*M*) [2]				
0	91.2±1.7	10.2 b	7.5 b	4.8 c
0.5	85.0±1.2	14.1 a	8.3 a	5.4 b
5	90.0±1.9	16.7 a	8.3 a	5.6 ab
10	92.5±1.3	13.7 a	8.5 a	6.2 a
50	90.0±2.7	12.6 ab	8.1 ab	5.7 ab
100	90.0±1.9	12.2 ab	8.1 ab	5.8 ab
PGPR strains				
G20-18	97.5±0.9	14.4 a	9.1 a	6.2 a
G8-32	95.0±1.8	13.9 a	8.8 a	6.3 a
63-28	95.0±1.8	13.4 ab	8.2 ab	6.1 a

[1] Means and standard errors. Percent germination was calculated for each GP with five seeds.

[2] Concentration of Z added to GP.

[3] Seeds were inoculated by soaking in bacterial suspensions for 3 h.

Data are means of three experiments with 10 replicate GP. Seedlings were harvested at 10 d.a.p. Means followed by the same letters are similar as determined by the Tukey pairwise comparison at the rejection level P=0.05.

Radish seeds soaked for 3 h in suspensions of strains G20-18WT, G8-32 and 63-28 containing 10^9 cfu mL^{-1} had bacterial densities before planting of Log 8.1, 8.2 and 8.6 cfu GP^{-1}, respectively. Root colonization by strains G20-18WT, G8-32 and 63-28 of radish seedlings grown for 10 d in GP were Log 8.2, 8.4 and 8.7 cfu GP^{-1}, respectively. No differences were observed among strains.

4.2.2.1.2.2. Combinations of IPA, Z and DHZR.

Total amounts of cytokinins estimated, as [IPA+ZR+DHZR] in the rhizosphere of non-inoculated seedlings and those inoculated with G20-18WT were 39.0 and 166.3 pmol GP^{-1}, respectively (Table 4.20). The rhizospheres of radish inoculated with G20-18WT had significantly higher amounts of each cytokinin than those of non-inoculated radish (P=0.05). The concentration and percent of each cytokinin differed significantly from all others, except in the case of ZR and DHZR in the rhizosphere of non-inoculated radish (P=0.05). As described for pure bacterial cultures (Table 4.3), IPA was also the most abundant cytokinin in the radish rhizosphere. The percentage of the amounts of IPA, ZR and DHZR to the total cytokinins produced by strain G20-18WT in the rhizosphere of radish 7 d.a.p was significantly different from that for non-inoculated radish (P=0.05).

Inoculation with strain G20-18WT or an exogenous supply of [IPA+Z+DHZR] mixtures prepared in the ratio of 50:10:40 had effects on the growth of radish seedlings in GP after 4 and 7 d (Figs. 4.16, 4.17 and 4.18). Root

length increased significantly with an exogenous supply of cytokinins higher than 0.5 nmol GP^{-1} when the seedlings grew for 4 d (P=0.05) (Fig. 4.16). However, when radish seedlings were grown for 7 d, cytokinin concentrations higher than 0.1 nmol GP^{-1} increased root length. Plants inoculated with G20-18WT had significantly greater root length than the control plants by 44 and 56 % at 4 and 7 d.a.p, respectively (P=0.05).

Table 4.20. Cytokinin production by *P. fluorescens* strain G20-18WT in the rhizosphere of radish cv. Cherry Belle growing in growth pouches.

	pmol GP^{-1} (1)		% of [IPA+ZR+DHZR] (2)	
Cytokinins	Non-inoculated	Inoculated	Non-inoculated	Inoculated
IPA	30.6±3.1	85.3±3.5	79	51
ZR	2.1±0.7	16.1±0.1	5	10
DHZR	6.3±0.4	64.9±0.9	16	39
LSD (3) $_{P=0.05)}$	5.4		1.7	

(1) Means and standards errors.

(2) Percent of total estimated cytokinins.

(3) LSD: Least significant difference using Tukey's test at the rejection level P=0.05.
Data are means of thee experiments with 10 replicate GP. GP were filled with 40 mL of MM+Gl. Seeds were soaked in 0.1 M MgSO4 solution or bacterial suspension for 3 h. Supernatants of radish rhizospheres were obtained after 7 d and immunoassayed for cytokinins.

Shoot length increased significantly with an exogenous supply of cytokinins higher than 0.05 nmol GP^{-1} when the seedlings grew for both 4 and 7 d.a.p (Fig. 4.17) (P=0.05). Shoot length of plants inoculated with G20-18WT was significantly greater than control plants by 46 and 31 % at 4 and 7 d.a.p, respectively (P=0.05).

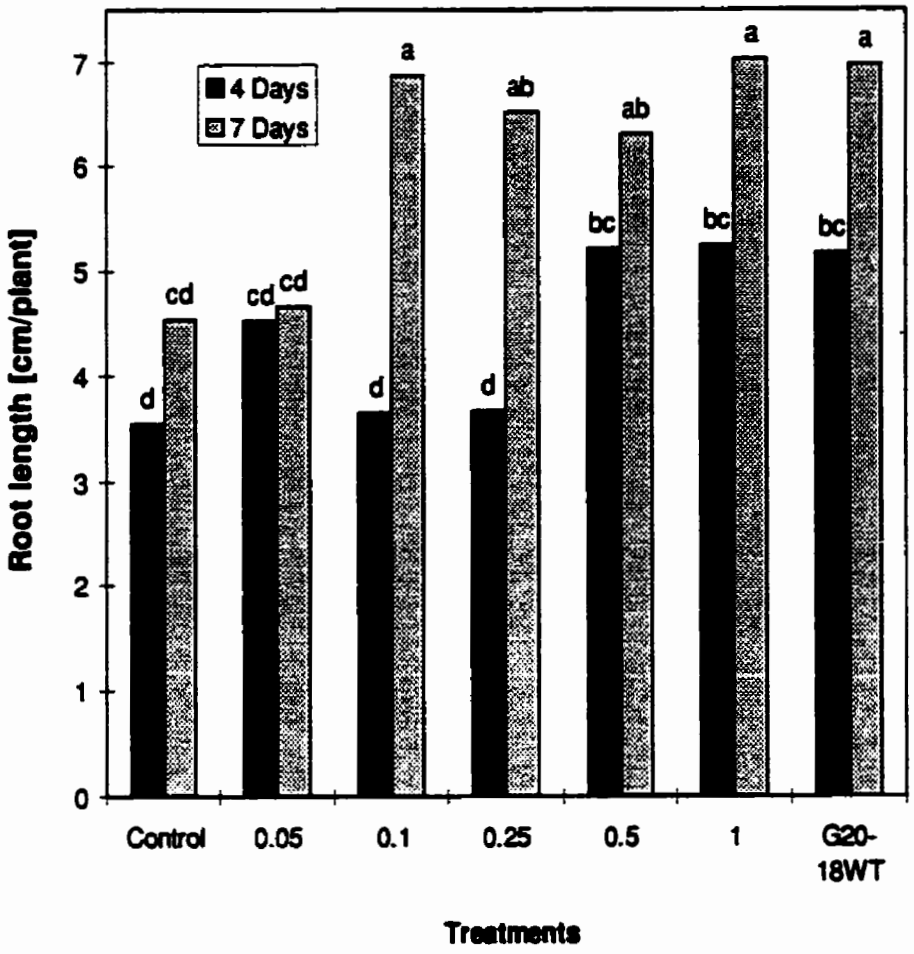


Figure 4.16. Effects of *P. fluorescens* strain G20-18WT and exogenous supply of cytokinin mixtures on root length of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments with 10 replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. Each cytokinin concentration in nmol GP^{-1} is a combination of IPA, ZR and DHZR in a ratio of 50:10:40, w/v.

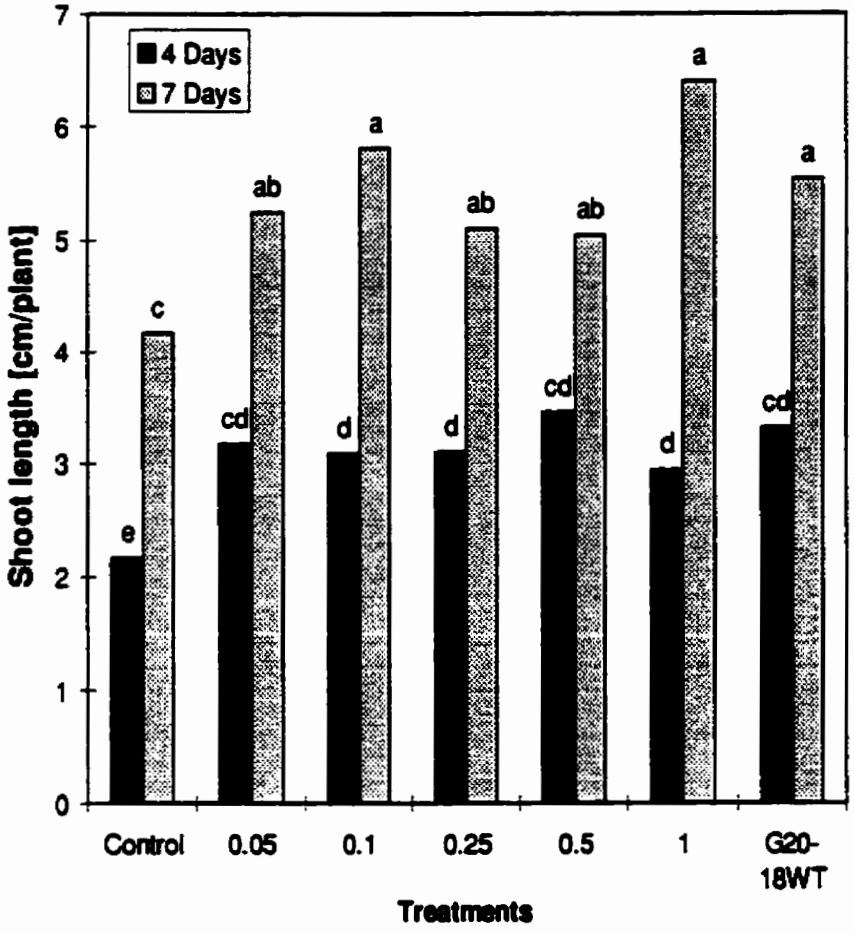


Figure 4.17. Effects of *P. fluorescens* strain G20-18WT and exogenous supply of cytokinin mixtures on shoot length of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments with 10 replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. Each cytokinin concentration in nmol GP^{-1} is a combination of IPA, ZR and DHZR in a ratio of 50:10:40, w/v.

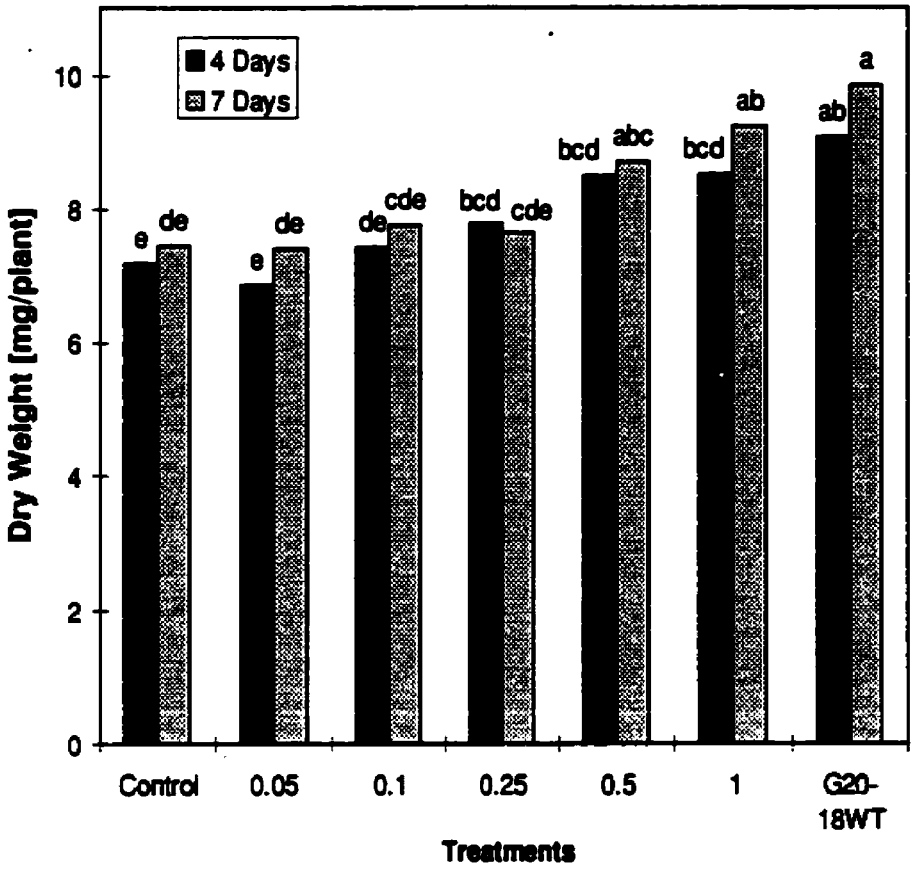


Figure 4.18. Effects of *P. fluorescens* strain G20-18WT and exogenous supply of cytokinin mixtures on dry weight of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments with 10 replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. Each cytokinin concentration in nmol GP^{-1} is a combination of IPA, ZR and DHZR in a ratio of 50:10:40, w/v.

Dry weights per plant increased with increasing concentrations of cytokinins (Fig. 4.18). The correlation between dry weight and cytokinin concentrations was r^2=0.90, (n=126; *P*=0.05). Radish seedlings inoculated with G20-18WT had the highest dry weight which was not significantly different from the dry weight of seedlings supplied with cytokinin concentrations higher than 0.25 and 0.5 nmol GP^{-1}, after 4 and 7 d.a.p, respectively (*P*=0.05). Inoculation with strain G20-18WT significantly increased dry weights by 30 and 21% at 4 and 7 d.a.p, respectively (Fig. 4.18) (*P*=0.05). Dry weight, root and shoot length of radish grown in GP increased significantly at 4 and 7 d.a.p when cytokinins were supplied exogenously or when seeds were inoculated with strain G20-18WT (*P*=0.05).

4.2.2.1.3. Study of cytokinin production by *P. fluorescens* strain G20-18WT and selected mutants as pure cultures and in association with radish plants.

When radish was grown in GP and inoculated with *P. fluorescens* strain G20-18WT and selected mutants, root lengths of 4 and 7-d old plants inoculated with G20-18WT were 1.9 and 2.5 fold higher, respectively than the control, (*P*=0.05) (Fig. 4.19). Strain RIF significantly increased root length of 7-d old plants by 1.5 fold over that of the control but its effect was significantly lower than that of strain G20-18WT (*P*=0.05). Root length of seedlings inoculated with mutants CNT1 and CNT2 did not differ from the control (Fig. 4.19). Shoot lengths of radish were significantly increased only by strain G20-18WT after both 4 and 7 d.a.p. (*P*=0.05) and these were 1.3 and 1.2 fold greater than the

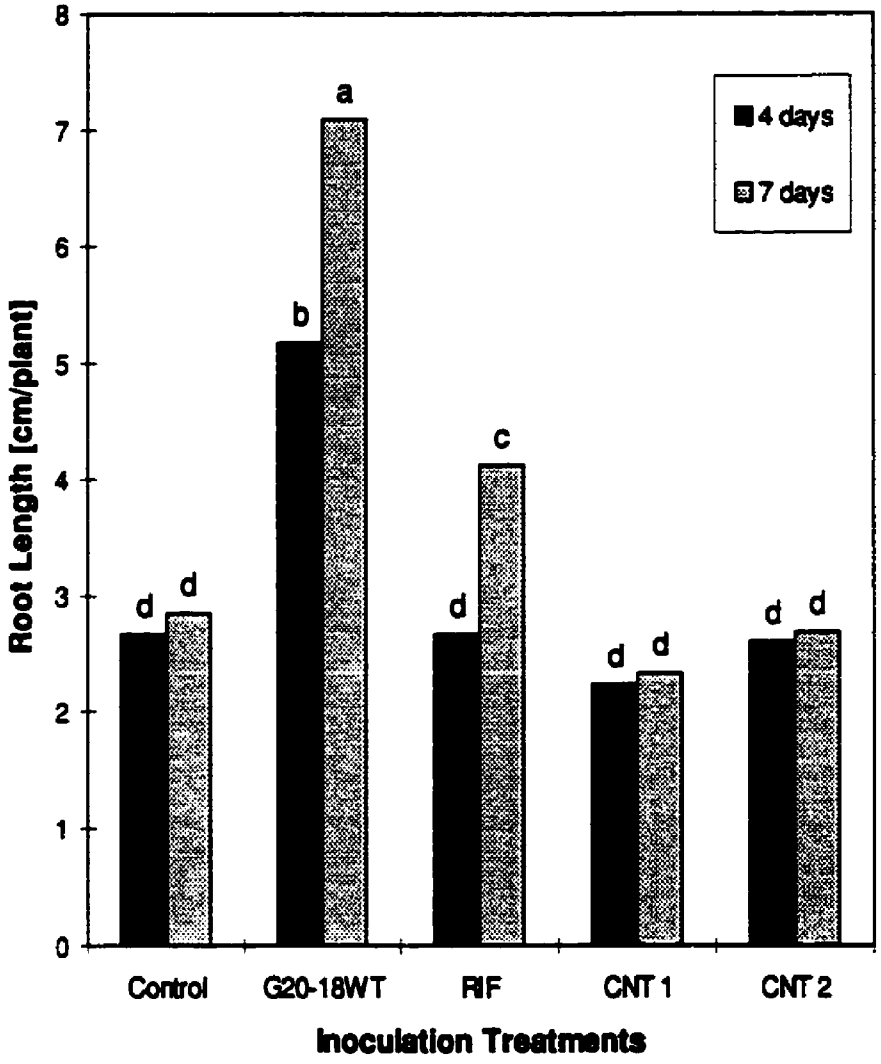


Figure 4.19. Effects of *P. fluorescens* strain G20-18WT and selected mutants on root length of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments with 10 replicate GP. Bars with the same letter indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

control, respectively (Fig. 4.20). Only plants inoculated with G20-18WT had significant increases in biomass at both 4 and 7 d.a.p (P=0.05) and these were 1.5 and 2.1 fold greater than the control, respectively (Fig. 4.21).

Data for IPA, ZR and DHZR production by strain G20-18WT and selected mutants growing in GP for 4 and 7 d.a.p were expressed as pmol GP^{-1} to allow the comparison of cytokinin production by pure cultures, sterile radish roots and radish inoculated with PGPR strains (Figs. 4.22, 4.23 and 4.24). The three cytokinins were detected in sterile radish rhizospheres and the total concentration of IPA+ZR+DHZR estimated at 4 d.a.p was 32.6 pmol GP^{-1} with a ratio of IPA:ZR:DHZR equal to 75:10:15. Cytokinin concentrations increased 1.5-fold for both IPA and DHZR and decreased 1.8-fold for ZR at 7 d.a.p giving a ratio of IPA:ZR:DHZR equal to 80:4:16. (Figs. 4.22, 4.23 and 4.24). Regardless of the treatments, IPA was the most abundant cytokinin and average IPA concentrations were always higher at 7 d.a.p. (47.4 pmol GP^{-1}) than at 4 d.a.p. (23.1 pmol GP^{-1}) (P=0.05). After 7 d.a.p. in pure culture, IPA production by strain G20-18WT was 2.9, 2.1 and 4.1 fold greater than that by mutants RIF, CNT1 and CNT2, respectively. With the exception of radish inoculated with strain RIF, radish inoculated with the mutants always had significantly less IPA than that with strain G20-18WT (Fig. 4.22) (P=0.05).

Radish GP inoculated with G20-18WT, RIF and CNT1 had significantly more ZR than sterile radish GP at 7 d.a.p. (Fig. 4.23) (P=0.05). Average ZR concentrations in pure cultures were 12.0 and 6.0 pmol GP^{-1} after 4 and 7 d.a.p,

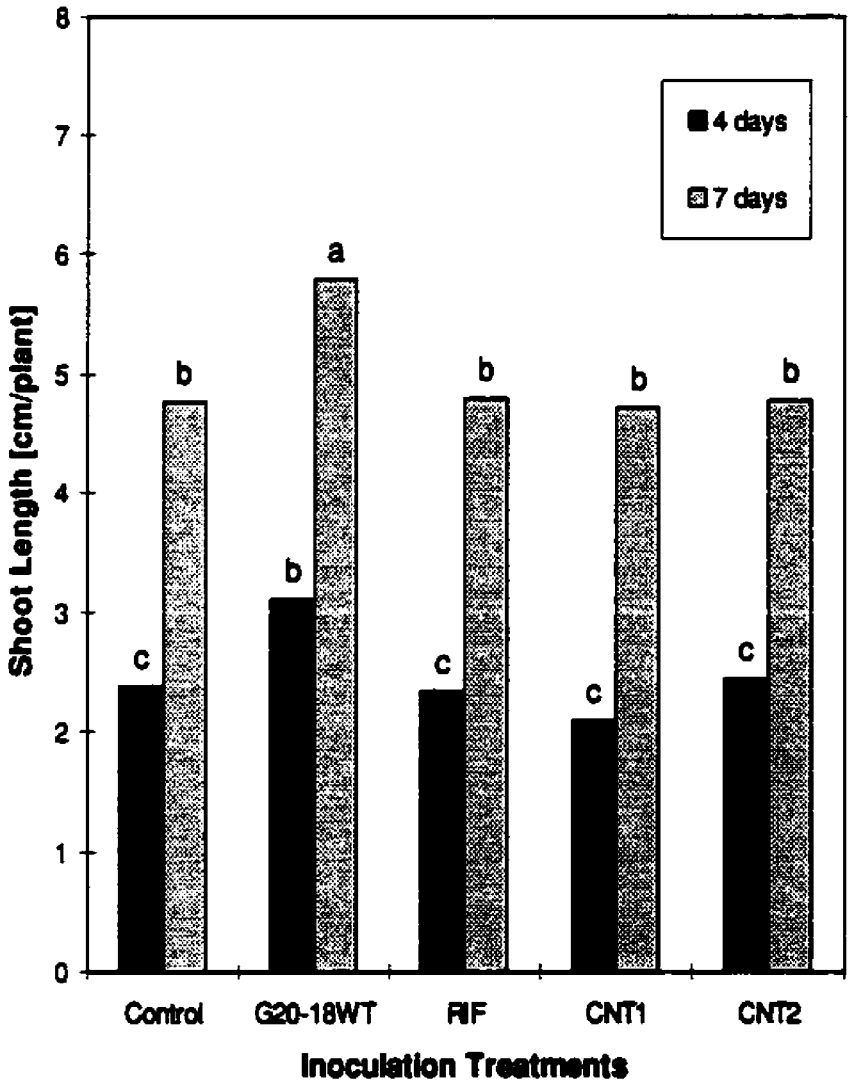


Figure 4.20. Effects of *P. fluorescens* strain G20-18WT and selected mutants on shoot length of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments with 10 replicate GP. Bars with the same letter indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

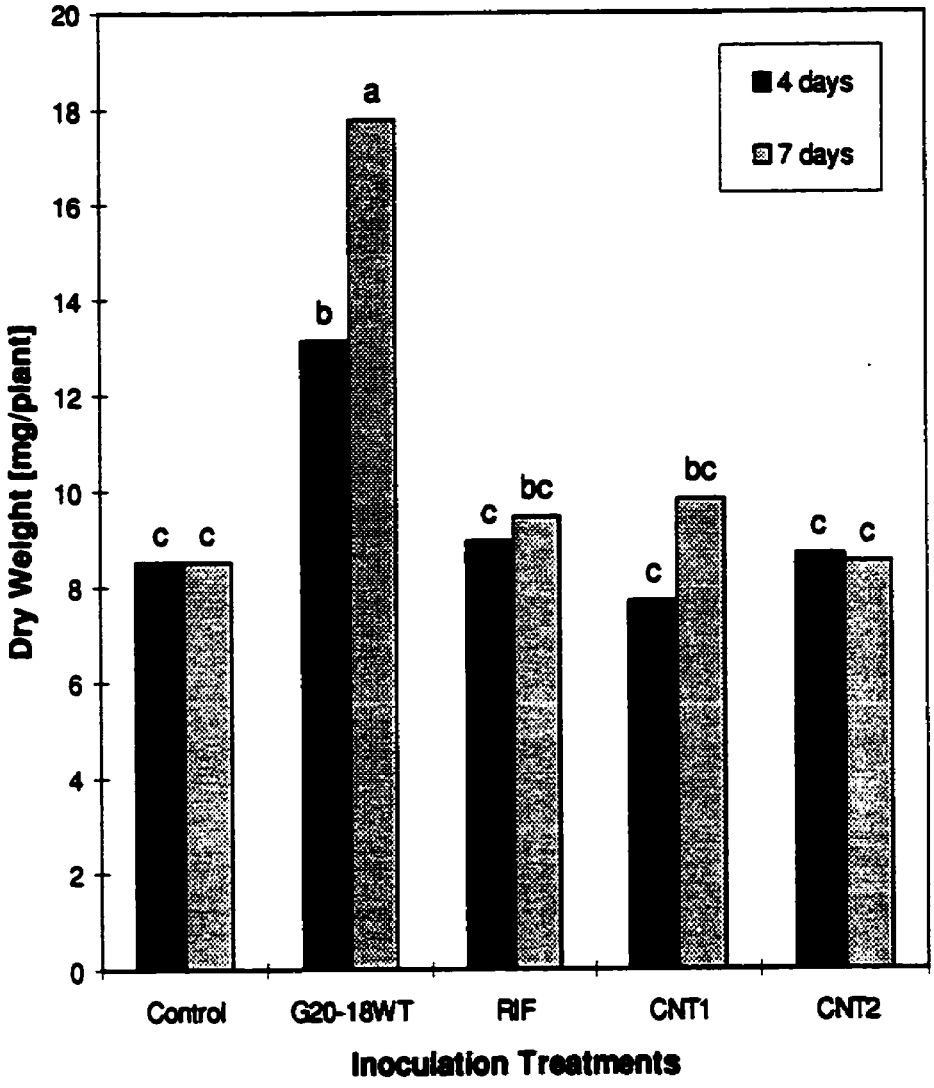


Figure 4.21. Effects of *P. fluorescens* strain G20-18WT and selected mutants on dry weight of radish cv. Cherry Belle seedlings grown in growth pouches.

Bars are means of three experiments through 10 replicate GP. Bars with the same letter indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

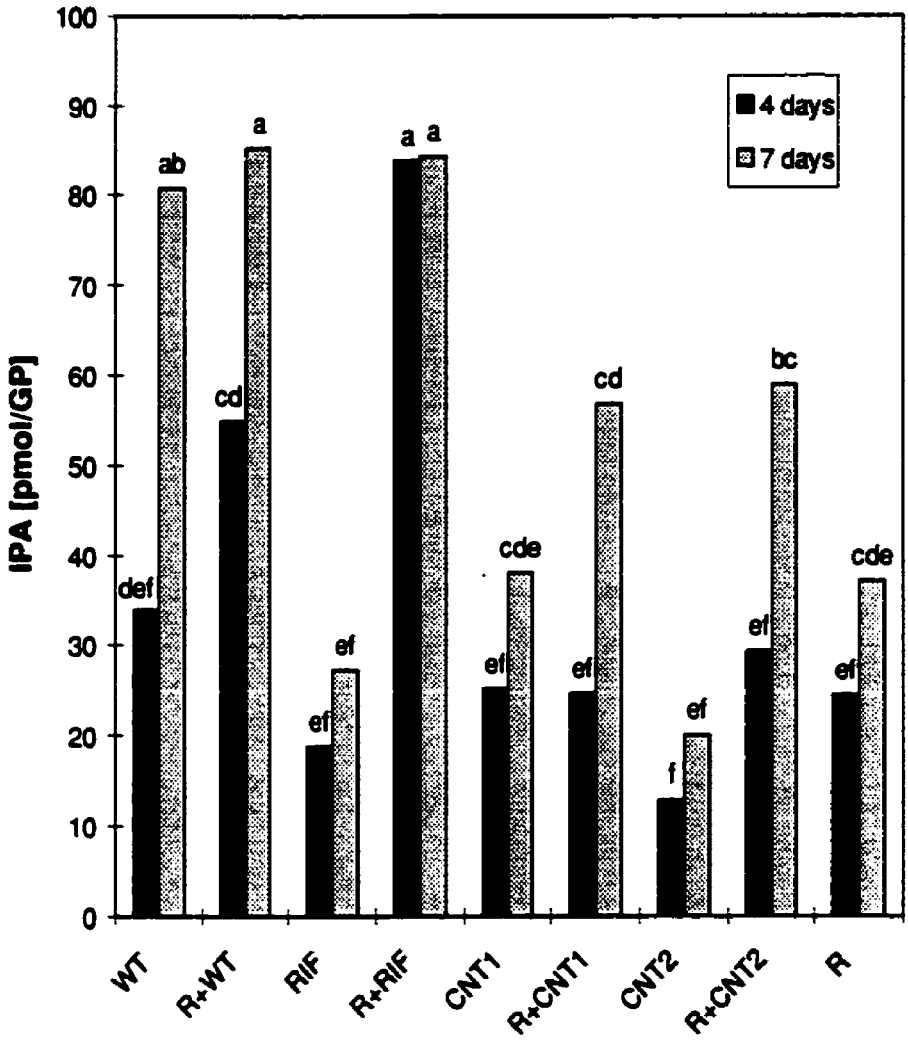


Figure 4.22. Concentrations of IPA in growth pouches inoculated with *P. fluorescens* strain G20-18WT and selected mutants in pure cultures and in the rhizosphere of radish cv. Cherry Belle.
WT (strain G20-18WT), RIF, CNT1 and CNT2: indicate GP with pure cultures of the respective PGPR strain. R: indicate GP with non-inoculated radish, R+WT, R+RIF, R+CNT1 and R+CNT2: indicate GP with respective PGPR strain and radish. Bars are means of three experiments with four replicate GP.

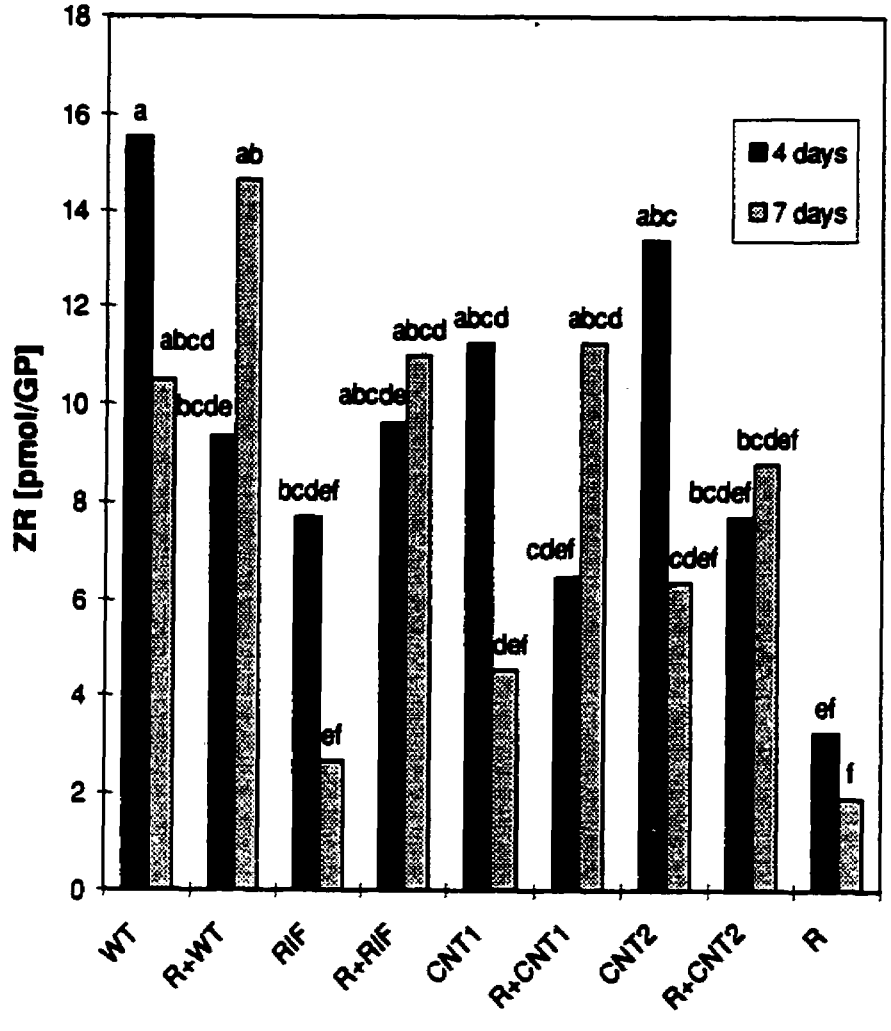


Figure 4.23. Concentrations of ZR in growth pouches inoculated with *P. fluorescens* strain G20-18WT and selected mutants in pure cultures and in the rhizosphere of radish cv. Cherry Belle.
WT (strain G20-18WT), RIF, CNT1 and CNT2: indicate GP with pure cultures of the respective PGPR strain. R: non-inoculated radish, R+WT, R+RIF, R+CNT1 and R+CNT2: indicate GP with respective PGPR strain and radish. Bars are means of three experiments with four replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

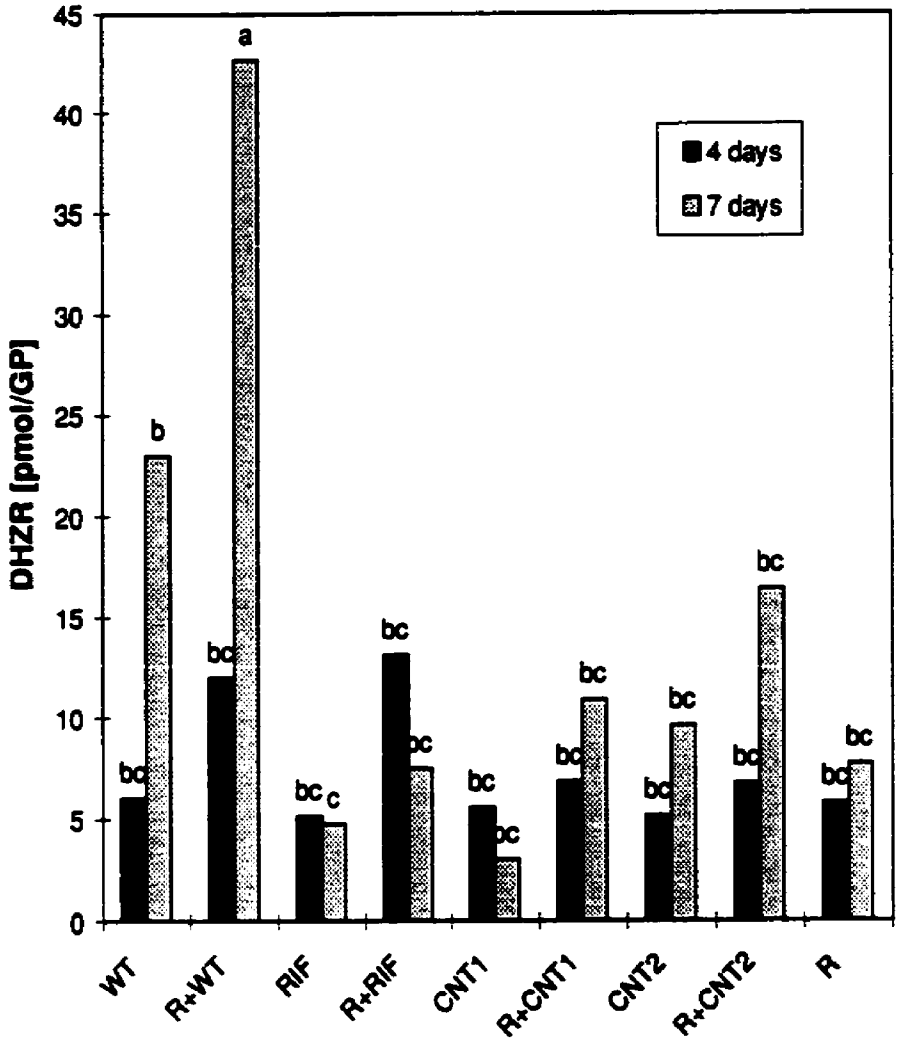


Figure 4.24. Concentrations of DHZR in growth pouches inoculated with *P. fluorescens* strain G20-18WT and selected mutants growing as pure cultures and in the rhizosphere of radish cv. Cherry Belle.
WT (strain G20-18WT), RIF, CNT1 and CNT2: indicate GP with pure cultures of the respective PGPR strain. R: non-inoculated radish, R+WT, R+RIF, R+CNT1 and R+CNT2: indicate GP with respective strain and radish. Bars are means of three experiments with four replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

respectively and these were significantly different (P=0.05). The concentration of DHZR in 7-d GP of radish inoculated with G20-18WT was 1.9 fold higher than that in GP with pure cultures of this strain (Fig. 4.24) (P=0.05). No differences among treatments were observed at 4 d.a.p. Average concentrations of DHZR over all treatments at 4 and 7 d.a.p. were 4.7 and 11.3 pmol GP^{-1}, respectively and these were significantly different (P=0.05). No differences were observed in the concentrations of IAA in GP containing pure cultures of strain G20-18WT, sterile radish or in radish inoculated with G20-18WT (Fig. 4.25). The average amount of IAA present in the GP was 146.7 nmol GP^{-1}.

Significant differences among strains were observed when the amounts of cytokinins in radish rhizospheres were related to the dry weight of the plants grown in the GP (Table 4.21) (P=0.05). The concentrations of IPA, ZR and DHZR per g dry weight in rhizospheres inoculated with transconjugants were similar to those for sterile radish rhizospheres at 4 and 7 d.a.p. The rhizosphere of plants inoculated with G20-18WT and RIF had significantly higher IPA per g dry weight than sterile radish at 4 and 7 d.a.p (P=0.05). Plants inoculated with G20-18WT had significantly higher ZR and DHZR per g dry weight than sterile radish rhizospheres at 7 d.a.p (P=0.05). Radish rhizospheres inoculated with G20-18WT had significantly higher DHZR per g dry weight than those inoculated with the mutant strains (P=0.05).

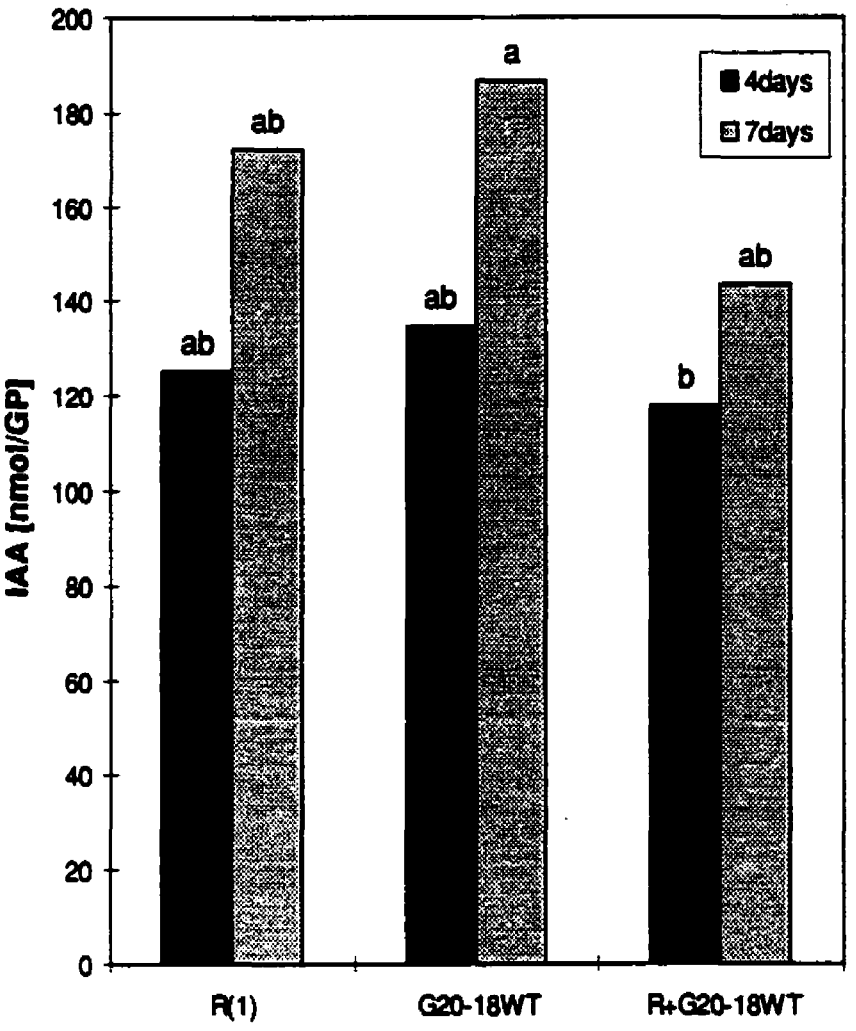


Figure 4.25. Concentrations of IAA in growth pouches inoculated with *P. fluorescens* strain G20-18WT growing as pure cultures and in the rhizosphere of radish cv. Cherry Belle.

Bars are means of three experiments with four replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. R(1), indicates non-inoculated radish seedlings.

Table 4.21. Effect of bacterial inoculation on concentrations in the rhizosphere of IPA, ZR and DHZR per g dry weight of plants grown for 4 and 7 d in growth pouches.

Treatments	Cytokinin Concentrations at d.a.p. (pmol g^{-1} plant dry weight) [(1)]					
	IPA		ZR		DHZR	
	4	7	4	7	4	7
Radish [R]	2.91d	4.29cd	0.39b	0.33b	0.67b	0.92b
R+G20-18WT	8.19ab	10.63a	1.12ab	2.13a	1.41ab	5.12a
R+RIF	6.23abc	9.55a	0.97ab	1.37ab	0.98b	0.85b
R+CNT1	4.29d	7.40abc	0.75b	1.37ab	0.81b	1.23b
R+CNT2	4.54bcd	8.38abc	0.69b	0.94ab	0.87b	2.60b

[(1)] Data are means of three experiments. Means followed by the same letter/s are not different as determined by Tukey's test at the rejection level P=0.05 for each cytokinin and sampling date.

Mean initial bacterial numbers in GP were Log 8.9, 8.8, 9.1 and 9.0 cfu GP^{-1} for strains G20-18WT, RIF, CNT1 and CNT2, respectively. The presence of a plant significantly reduced numbers of G20-18WT and RIF compared to pure cultures at 4 d.a.p, but bacterial numbers increased significantly relative to the pure cultures at 7 d.a.p. (P=0.05) (Fig. 4.26). Numbers of mutant CNT1 were significantly higher in pure cultures than in the rhizosphere of radish (P=0.05). This indicates that the plant had a negative effect on the survival of this mutant. Similarly, the survival of mutant CNT2 in the rhizosphere of radish was lower than in pure cultures at 4 d.a.p (P=0.05). Numbers of both transconjugants were significantly lower than those of G20-18WT and RIF, except for 7-d pure cultures of CNT1 (P=0.05).

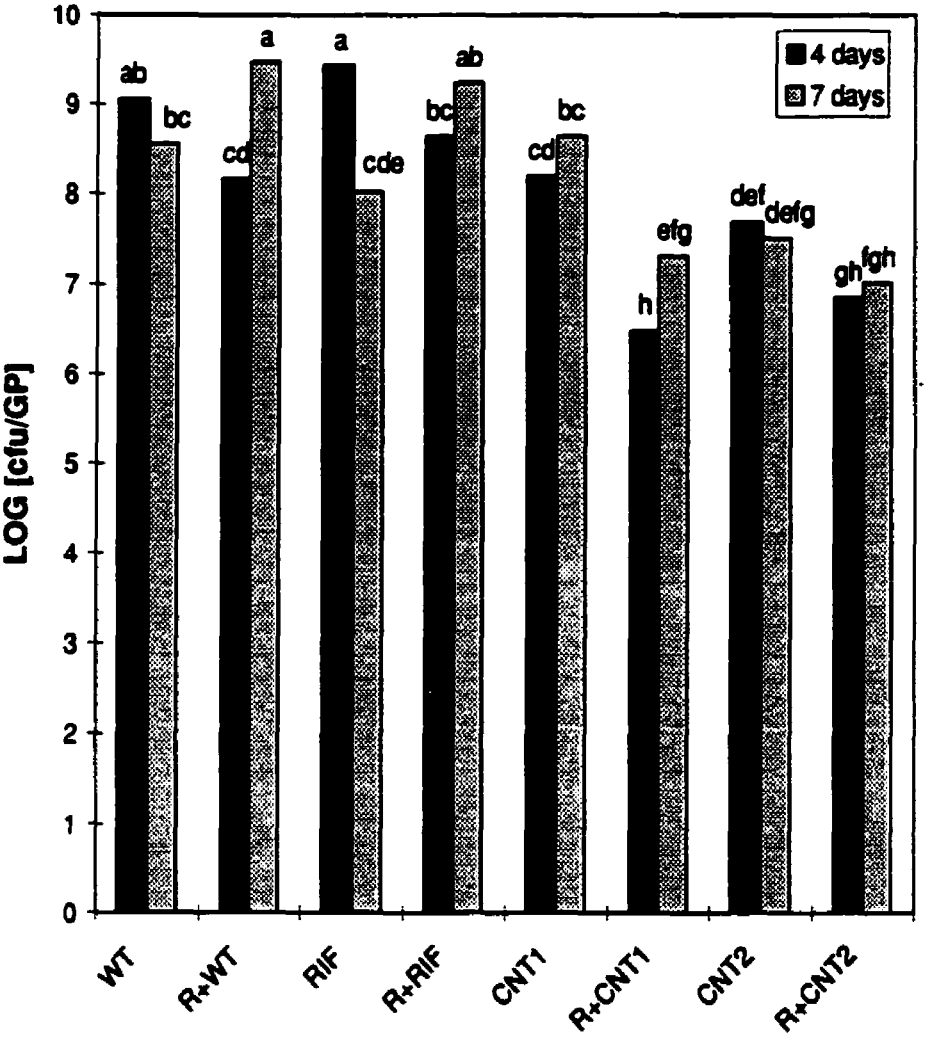


Figure 4.26. Survival of *P. fluorescens* strain G20-18WT and selected mutants growing in growth pouches as pure cultures and in rhizospheres of radish cv. Cherry Belle.

WT (strain G20-18WT), RIF, CNT1 and CNT2: indicate GP with pure cultures of the respective PGPR strain. R+WT, R+RIF, R+CNT1 and R+CNT2: indicate GP with respective strain and radish. Bars are means of three experiments with three replicate GP. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

4.2.2.2. Small pots

4.2.2.2.1. Screening of PGPR strains

The effects of PGPR strains on emergence, root and shoot biomass of radish cv. Cherry Belle grown in small pots under greenhouse conditions are shown in Table 4.22. Emergence of radish was increased by PGPR strains only at 6 d.a.p. Inoculation with strains G20-18WT, G8-32 and 63-28 significantly increased emergence by 16, 11 and 11%, respectively (P=0.05). Strains GR12-2 and Ral-3 did not differ from the control. Root and shoot biomass of radish seedlings inoculated with strain G20-18WT were significantly increased by 39 and 11%, respectively (P=0.05). Other strains did not show any significant effect on root and shoot biomass.

Table 4.22. Effects of PGPR strains on emergence, root and shoot biomass of radish cv. Cherry Belle grown in small pots under greenhouse conditions.

PGPR Strains	% Emergence [1] at days after planting		Biomass [2] (mg plant^{-1})	
	4	6	Root	Shoot
Control	58.8 a	68.7 b	11.0 b	74.4 b
G20-18WT	67.7 a	84.4 a	15.3 a	82.6 a
GR12-2	65.6 a	78.1 ab	11.5 b	78.7 ab
G8-32	65.3 a	79.5 a	14.5 ab	80.4 ab
63-28	66.6 a	79.9 a	14.3 ab	80.9 ab
Ral 3	58.3 a	68.4 b	11.1 b	74.9 b

Means of three experiments with three replicates. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

[1] Each replicate consisted of a tray with 48 pots planted with two seeds.

[2] Plants were harvested at 18 d.a.p.

Radish roots inoculated with strain 63-28 had the highest bacterial numbers, which were significantly (P=0.05) greater than those inoculated with strains G20-18WT, GR12-2 and Ral-3 (Fig. 4.27). There were no significant differences between bacterial numbers on seeds and roots of plants at 18 d.a.p of each strain (P=0.05).

4.2.2.2.2. Dose-response of exogenously-applied cytokinins

The percentage of emerged plants was similarly increased by PGPR strains and exogenous applications of Z (Table 4.23). On average, emergence of inoculated seedlings and those supplied with Z was significantly higher than the control by 19 and 8%, respectively (P=0.05). On average, PGPR strains significantly increased root and shoot biomass by 33 and 61%, respectively (P=0.05). Relative surface area of the roots (RRSA) of plants inoculated with G20-18WT was significantly different from that of plants inoculated with 63-28 (P=0.05) (Table 4.23).

Exogenous applications of Z significantly increased root and shoot biomass and RRSA of radish seedlings (P=0.05) (Table 4.23). Root biomass of seedlings supplied with 5 nM Z was significantly higher than 0.5 and 100 nM applications (P=0.05). Exogenous applications of Z ranging between 0.5 and 50 nM had similar effects on shoot biomass and RRSA of radish seedlings. Shoot biomass of radish seedlings supplied with 100 nM Z was significantly lower than that of seedlings supplied with 1 and 5 nM Z, but significantly (P=0.05) higher than that of controls (P=0.05) (Table 4.23).

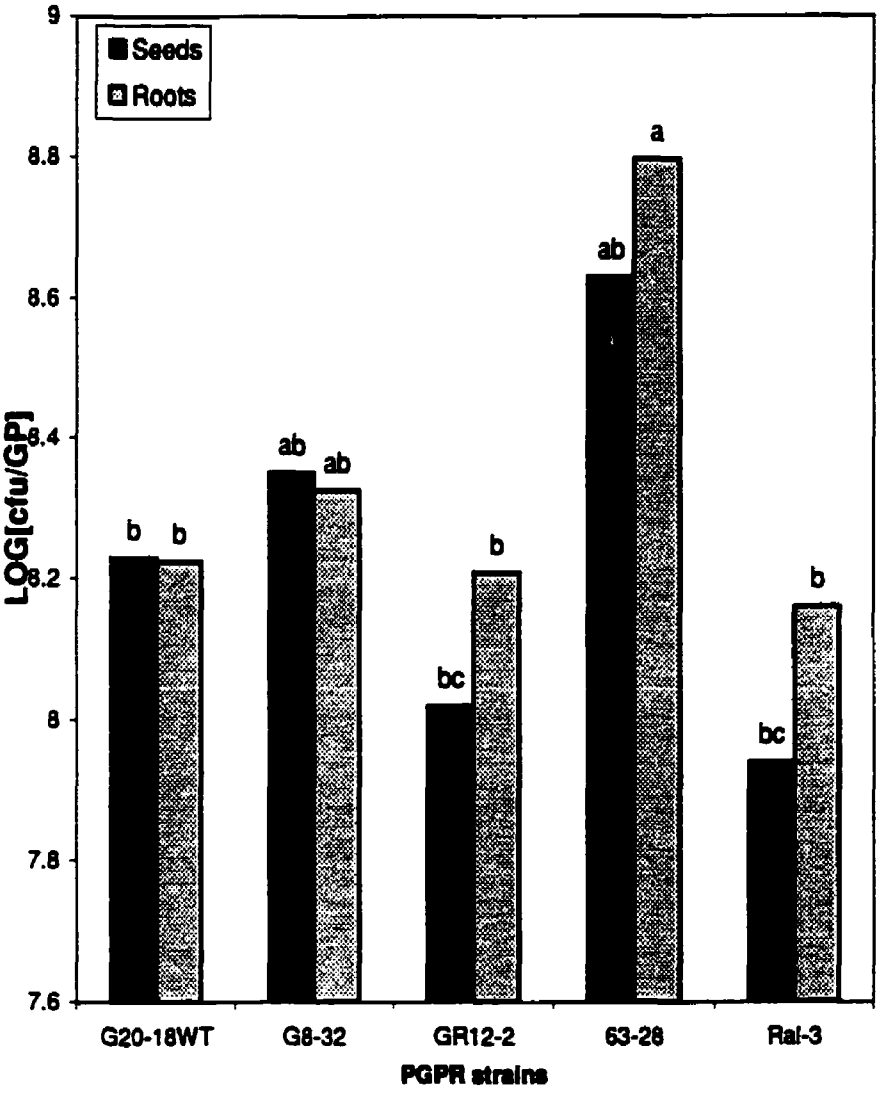


Figure 4.27. Bacterial numbers on seeds and roots of radish cv. Cherry Belle grown in small pots and inoculated with selected PGPR strains.
Bars are means of three experiments with three replicate GP. Means with the same letter/s are not different as determined by Tukey's test at the rejection level P=0.05. No bacteria were detected from non-inoculated seedlings. Seeds represent bacterial numbers on inoculated seeds before planting and Roots, the number of bacteria on roots of 18 d-old plants.

Table 4.23. Effects of three *Pseudomonas* PGPR or exogenous applications of Z on emergence, root and shoot biomass and relative root surface area (RRSA) of radish cv. Cherry Belle grown in small pots under greenhouse conditions.

Treatments	Emerged Plants[1] (%)	Biomass (mg plant^{-1})		RRSA (mg plant^{-1})[2]
		Root	Shoot	
Control	63.5 c	12.6 d	62 .0 d	157.2 d
PGPR				
G20-18WT	84.9 a	18.0 ab	100.2 ab	275.4 a
G8-32	77.6 ab	15.0 bc	95.8 bc	237.2 ab
63-28	83.3 a	17.3 ab	105.1 ab	181.7 bc
Z (n*M*)				
0.5	71.9 ab	13.1 bc	118.2 ab	262.1 a
1	70.8 ab	15.6 ab	133.8 a	253.2 a
5	70.0 ab	19.2 a	136.6 a	277.7 a
10	70.8 ab	16.7 ab	118.9 ab	304.5 a
50	77.1 ab	15.6 ab	110.8 ab	n.d.[3]
100	68.8 ab	13.6 bc	96.9 bc	n.d.

Data are means of three experiments with three replicates. Plants were harvested at 18 d.a.p. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

[1] Each replicate consisted of a tray with 48 pots planted with two seeds.

[2] Roots were immersed in $Ca(NO_3)_2$ solution as described by Carley and Watson (1966).

[3] not determined.

In another set of experiments, inoculation with PGPR strains increased the percentage of emerged plants but no differences were observed among strains (Table 4.24). Exogenous applications of 0.5 and 5 n*M* DHZR significantly increased radish emergence by 41 and 17%, respectively (P=0.05). PGPR inoculation or addition of DHZR significantly (P=0.05) increased root and shoot biomass of radish seedlings. No differences were observed among strains and DHZR concentrations (Table 4.24).

Table 4.24. Effects of three *Pseudomonas* PGPR and exogenous applications of DHZR on emergence, root and shoot biomass and RRSA of radish cv. Cherry Belle grown in small pots under greenhouse conditions.

Treatments	Emerged Plants[1] (%)	Biomass (mg plant^{-1})		RRSA (mg plant^{-1})[2]
		Root	Shoot	
Control	58.3 c	13.5 b	66.2 b	150.3 c
PGPR				
G20-18WT	72.2 ab	17.7 a	119.9 a	193.2 b
G8-32	69.5 b	16.9 a	114.7 a	188.4 b
63-28	69.5 b	16.4 a	113.0 a	185.1 b
DHZR (n*M*)				
0.5	81.9 a	17.2 a	107.4 a	210.2 a
5	68.1 b	19.6 a	93.4 a	212.3 a
10	63.9 bc	17.8 a	113.2 a	215.9 a
50	62.5 bc	17.9 a	102.3 a	211.8 a

Means of three experiments with three replicates. Plants were harvested at 18 d.a.p. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

[1] Each replicate consisted of a tray with 48 pots planted with two seeds.

[2] Roots were immersed in $Ca(NO_3)_2$ solution as described by Carley and Watson (1966).

The effect of exogenous applications of DHZR on RRSA was significantly higher than the effect of PGPR inoculation (P=0.05). On average, PGPR inoculation and exogenous applications of DHZR increased RRSA by 26% and 42%, respectively. However, no differences were observed among strains or DHZR concentrations.

Radish seeds soaked in suspensions of strains G20-18WT, G8-32 and 63-28 for 3 h had bacterial densities of Log 8.2, 8.3 and 8.6 cfu GP^{-1}, respectively. Root colonization at 18 d.a.p did not differ significantly among PGPR strains (P=0.05). Bacterial numbers on roots were Log 8.3, 8.4 and 8.7 cfu mg^{-1} dry

weight of roots for strains G20-18WT, G8-32 and 63-28, respectively and they were similar to those on seeds inoculated with each PGPR strain.

Table 4.25 shows the effect of strain G20-18WT or exogenous applications of Z lower than 1 n*M* on shoot biomass and RRSA of radish seedlings. All concentrations of Z had an effect on shoot biomass and RRSA. Shoot biomass of radish seedlings was significantly (P=0.05) increased by 31, 36 and 61% when concentration ranges of 0.05-01, 0.25-0.5 and 1 n*M* Z, respectively, were supplied exogenously. Concentration ranges of Z of 0.05-0.25 and 0.5-1 n*M* significantly increased RRSA by 31 and 55%, respectively (P=0.05). Strain G20-18WT significantly increased shoot biomass (58%) and RRSA (57%) (P=0.05) and its effects were similar to those produced by concentrations of Z equal to or higher than 0.25 and 0.5 n*M*, respectively. Radish seeds and roots had similar bacterial numbers as those estimated for strain G20-18WT (Fig. 4.27).

Inoculation with G20-18WT significantly (P=0.05) increased the concentrations of IPA and DHZR present in root and shoot tissues of radish grown in greenhouse conditions in a different way than the exogenous supply of 0.5 n*M* Z (Table 4.26). The amounts of IPA in roots and shoots of plants inoculated with G20-18WT were 2.0 and 1.7 fold greater than the controls. Supply of 0.5 n*M* Z significantly increased the amount of IPA present in shoots up to 2.3 fold greater than the controls (P=0.05), but IPA concentrations in roots were similar to the controls (Table 4.26).

Table 4.25. Effects of *P. fluorescens* strain G20-18WT or exogenous applications of Z on shoot biomass and RRSA of radish cv. Cherry Belle grown in greenhouse conditions.

Treatments	Shoot Biomass (mg plant^{-1})	RRSA (mg plant^{-1}) (1)
Control	90.4 c	163.1 c
Z (n*M*)		
0.05	118.8 b	209.1 b
0.1	118.4 b	211.8 b
0.25	125.1 ab	217.8 b
0.5	128.1 ab	249.8 a
1	145.4 a	256.4 a
Strain		
G20-18WT	142.6 a	256.6 a

Means of three experiments with three replicates. Plants were harvested at 18 days after planting. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

(1) Roots were immersed in $Ca(NO_3)_2$ solution as described by Carley and Watson (1966).

Table 4.26. Concentrations of IPA, ZR and DHZR present in root and shoot tissue of cv. Cherry Belle grown in greenhouse conditions and supplied with Z or inoculated with *P. fluorescens* strain G20-18WT.

Treatments	Cytokinin in plant tissue [nmol g^{-1} fresh weight]					
	IPA		ZR		DHZR	
	Root	Shoot	Root	Shoot	Root	Shoot
Control	22.2 b	4.1 c	5.7 b	2.4 b	3.1 c	5.2 c
G20-18WT	44.3 a	7.1 b	8.3 a	5.2 a	4.2 b	11.9 a
0.5 n*M* Z	22.1 b	9.6 a	8.2 a	4.8 a	5.9 a	8.6 b
P	0.05	0.05	0.05	0.05	0.01	0.01

Means of three experiments with three replicates. Plants were harvested at 18 d.a.p. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the indicated rejection level.

Inoculation with G20-18WT or exogenous application of 0.5 n*M* Z significantly increased the concentrations of ZR present in both roots and shoots

by a similar order of magnitude, up to 1.5 and 2.1 fold greater than the controls (Table 4.26) (P=0.05).

The concentrations of DHZR present in roots of inoculated plants were significantly higher than in the controls (P=0.05), but they were significantly lower than those present in roots of plants exogenously supplied with 0.5 n*M* Z (P=0.05). However, plants inoculated with G20-18WT contained significantly more DHZR in their shoots than plants exogenously supplied with Z (P=0.05). The concentrations of DHZR in roots and shoots of plants inoculated with G20-18WT were 1.4 and 2.3 greater than the controls, respectively. The concentrations of DHZR in roots and shoots of plants supplied with 0.5 n*M* Z were 1.9 and 1.7 fold greater than the controls (Table 4.26).

4.2.2.2.3. Comparison of inoculation effects of *P. fluorescens* strain G20-18WT and selected mutants.

Emergence of radish plants was not affected by strain G20-18WT at 4 and 6 d.a.p (Table 4.27). Emergence of seedlings inoculated with mutants RIF, CNT1 and CNT2 at 4 d.a.p was significantly reduced by 11, 10 and 18%, respectively (P=0.05). No differences among strains were observed at 6 d.a.p. At 8 and 18 d.a.p the emergence of seedlings inoculated with the mutants was similar to the controls but emergence of seeds inoculated with G20-18WT was significantly higher than the controls by 12 and 8%, respectively (P=0.05) (Table 4.27).

Inoculation with strain G20-18WT significantly (P=0.05) increased shoot biomass and RRSA of radish plants by 49 and 27%, respectively (Table 4.28). Strain RIF significantly increased shoot biomass and RRSA of radish plants by

Table 4.27. Effects of P. fluorescens strain G20-18WT and selected mutants on emergence of radish cv. Cherry Belle grown in small pots under greenhouse conditions.

Strains	% Emerged plants at d.a.p. [1]			
	4	6	8	18
Control	50.0 a	63.3 a	69.4 b	78.3 b
G20-18WT	50.6 a	68.9 a	81.1 a	86.1 a
RIF	40.0 bc	67.2 a	76.7 ab	83.9 ab
CNT1	40.6 b	62.2 a	74.4 ab	78.9 ab
CNT2	32.2 c	62.8 a	73.3 b	82.2 ab

[1] Means of three experiments with three replicates. Each replicate consisted of a tray with 48 pots planted with two seeds. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

Table 4.28. Effects of *P. fluorescens* strain G20-18WT and selected mutants on shoot biomass, RRSA and chlorophyll units of radish cv. Cherry Belle grown in greenhouse conditions.

Strains	Shoot Biomass	RRSA [1]	Chlorophyll Units [2]	
	(mg plant^{-1})		Cotyledon	M.E.Leaf [3]
Control	61.5 c	129.8 c	21.1 b	38.4 a
G20-18WT	91.6 a	165.1 a	30.7 a	40.1 a
RIF	78.8 b	149.4 b	29.7 a	39.5 a
CNT1	65.2 c	121.3 c	16.2 b	40.9 a
CNT2	63.7 c	110.1 c	17.9 b	40.1 a

Means of three experiments with three replicates. Plants were harvested at 18 d.a.p. Values followed by the same letter/s indicate no significant differences between means as determined by the Tukey's test at the rejection level P=0.05.

[1] Roots were immersed in $Ca(NO_3)_2$ solution as described by Carley and Watson (1966).

[2] All plants were measured with a portable chlorophyll unit meter.

[3] Most expanded leaf of each plant.

28 and 15%, respectively (P=0.05). However, these effects were significantly (P=0.05) different from those observed for strain G20-18WT. Inoculation with

transconjugants CNT1 and CNT2 did not affect shoot biomass or RRSA of radish.

Although there were no differences among strains in the number of chlorophyll units of the most expanded leaves, significant differences among inoculation treatments were observed when the chlorophyll units of cotyledons were compared (Table 4.28) (P=0.05). Plants inoculated with strains G20-18WT and RIF had 46 and 41% higher numbers of active chlorophyll units in the cotyledons, respectively than the control (P=0.05).

Radish plants inoculated with strain G20-18WT had significantly higher amounts of IPA, ZR and DHZR in both roots and shoots than plants inoculated with transconjugants CNT1 and CNT2 (Table 4.29) (P=0.05). Roots of plants inoculated with G20-18WT had significantly increased concentrations of IPA, ZR and DHZR by 2.3, 2.6 and 1.4 fold over the controls, respectively (P=0.05). Shoots of plants inoculated with G20-18WT had significantly increased concentrations of IPA, ZR and DHZR by 1.6, 2.1 and 1.7 fold over the controls, respectively (P=0.05). Total amounts of cytokinins (IPA+ZR+DHZR) present in roots and shoots of radish inoculated with strain G20-18WT were 82.2 and 44.8 nmol g^{-1} fresh weight of plant tissue, respectively and these were significantly different (P=0.05). Total cytokinins present in plant tissues were correlated with shoot biomass and RRSA with correlation coefficients (P=0.05) equal to 0.90 and 0.92, respectively.

Initially all the strains had similar bacterial numbers on the seeds with an average Log 8.9 cfu $seed^{-1}$ (Fig. 4.28). Counts performed at 7 d.a.p showed

Table 4.29. Effects of *P. fluorescens* strain G20-18WT and selected mutants on the concentrations of IPA, ZR and DHZR present in root and shoot tissues of cv. Cherry Belle grown in greenhouse conditions.

Treatments	Cytokinin in plant tissue [nmol g^{-1} fresh weight] (1)					
	IPA		ZR		DHZR	
	Root	Shoot	Root	Shoot	Root	Shoot
Control	19.3 b	13.3 b	9.7 c	6.8 b	8.3 b	5.1 b
G20-18WT	45.2 c	21.8 a	25.2 a	14.4 a	11.8 a	8.6 a
RIF	21.2 b	10.4 b	19.5 ab	7.3 b	9.4 ab	8.1 a
CNT1	10.5 b	8.2 b	13.8 bc	5.6 b	7.9 b	3.1 b
CNT2	11.6 b	9.7 b	15.6 bc	5.1 b	7.8 b	3.2 b
P	0.01	0.01	0.01	0.01	0.05	0.01

Means of three experiments with three replicates. Plants were harvested at 18 d.a.p. Values followed by the same letter/s indicate no significant differences between means as determined by Tukey's test at the indicated rejection level.
(1) Data estimated using immunoassays.

that all the strains had similar levels of root colonization, which were similar to those counted on seeds. However, at 15 d.a.p root counts were significantly (P=0.05) lower than bacterial numbers on the seeds for the selected mutants.

4.2.2.3. Large Pots

The effects of PGPR strains on root, shoot and tuber biomass of radish cv. Cherry Belle grown in large pots under greenhouse conditions are shown in Table 4.30. Root, shoot and tuber biomass of plants inoculated with strain G20-18WT were 1.6, 1.3 and 1.2 fold greater than the control. They did not differ from those inoculated with strains GR12-2, G8-32 and 63-28. Dry weights of plants inoculated with these strains were similar to those inoculated with Ral-3, which was also similar to those of the control, but significantly lower

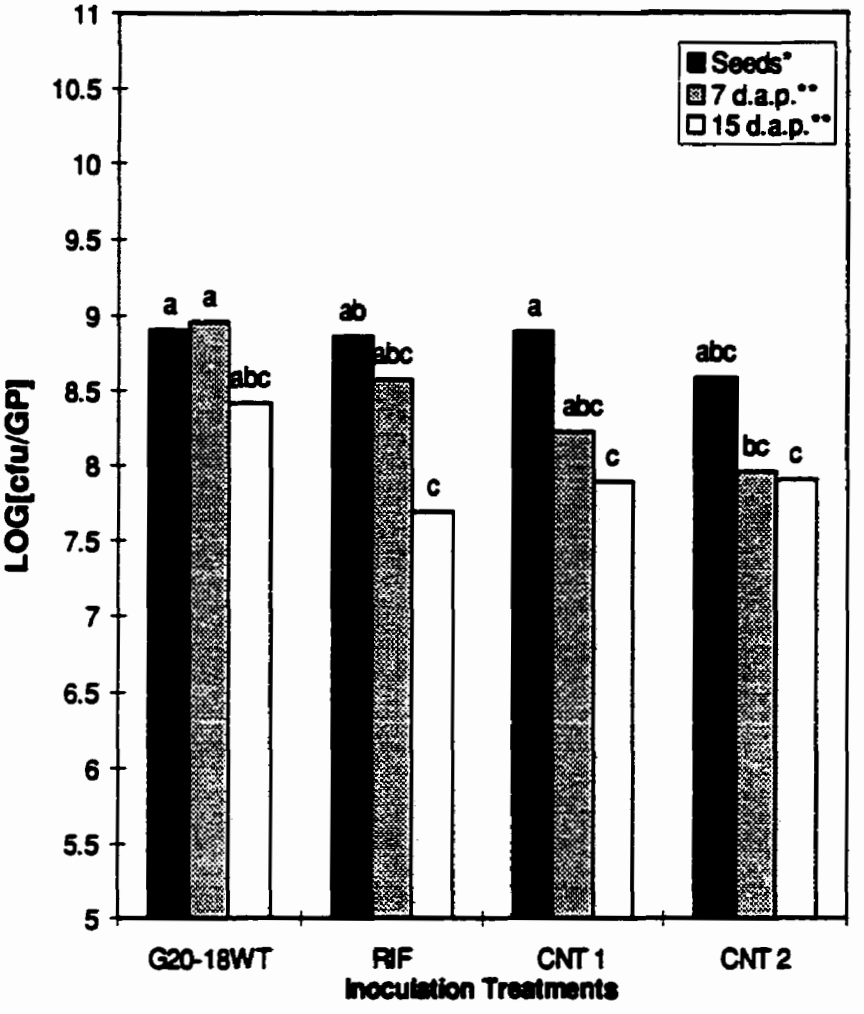


Figure 4.28. Bacterial numbers on seeds and roots of radish cv. Cherry Belle inoculated with *P. fluorescens* strain G20-18WT and selected mutants and grown in small pots.

Bars are means of three experiments with three replicates. Bars with the same letter/s above the bar indicate no significant differences between means as determined by Tukey's test at the rejection level (P=0.05). No bacteria were detected from non-inoculated seedlings. Seeds* represents bacterial numbers on inoculated seeds before planting. 7 d.a.p** and 15 d.a.p.** represent bacterial numbers on root at indicated d.a.p.

than those with G20-18WT. Percentage of water in the tubers before drying was similar for all treatments with an average of 93.6%.

Table 4.30. Effects of PGPR strains on root, shoot and tuber biomass of radish cv. Cherry Belle grown in large pots under greenhouse conditions.

Strains	Biomass [1] [mg plant^{-1}]		
	Root	Shoot	Tubers
Control	66.7 b [2]	705.4 c	1334.1 b
G20-18WT	106.2 a	929.5 a	1617.4 a
GR12-2	84.9 ab	911.8 ab	1586.7 ab
G8-32	97.4 ab	917.2 ab	1537.7 ab
63-28	92.6 ab	922.5 ab	1494.9 ab
Ral-3	65.4 b	767.4 bc	1330.3 b

[1] Plants were harvested 30 d.a.p. Means of 13 replicated large pots with three plants.
[2] Means followed by the same letter/s are similar as determined by Tukey's test at the rejection level P=0.05.

Strains differed in their ability to colonize radish seeds and roots (Fig. 4.29). Numbers of G20-18WT and Ral-3 were similar on seeds and roots and these were lower than those for strains G8-32 and 63-28 and higher than those for GR12-2. Bacterial numbers on roots were significantly lower than those on seeds (P=0.05).

The numbers of chlorophyll units in the most expanded leaf of plants inoculated with PGPR strains were similar to those measured in the most expanded leaf of uninoculated plants 20 d.a.p (Fig. 4.30). However, numbers of

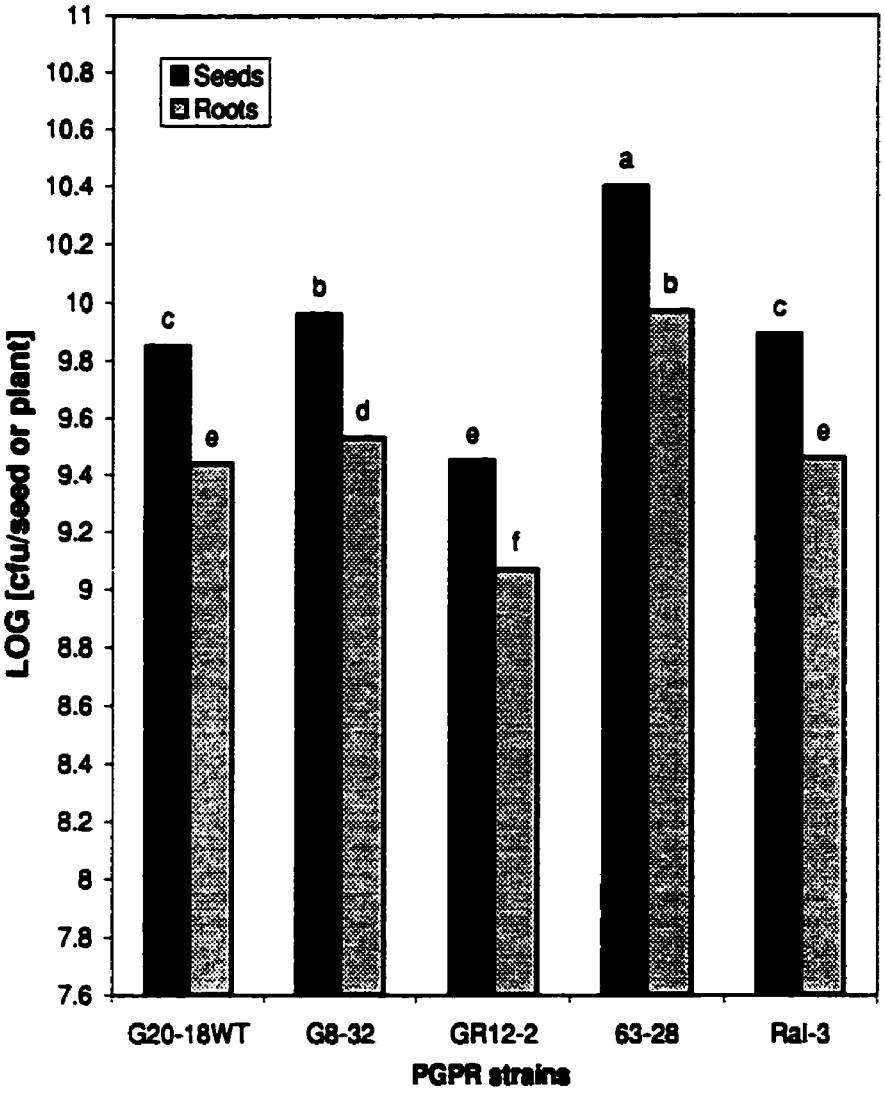


Figure 4.29. Bacterial numbers of PGPR strains on seeds and roots of radish cv. Cherry Belle grown in large pots under greenhouse conditions. Bars are means of three replicates. No bacteria were detected from non-inoculated roots. Seeds indicate bacterial numbers on inoculated seeds before planting. Roots indicate bacterial numbers on roots of 5 d-old seedlings. Bars with the same letter indicate no significant differences as determined by Tukey's test at the rejection level P=0.05.

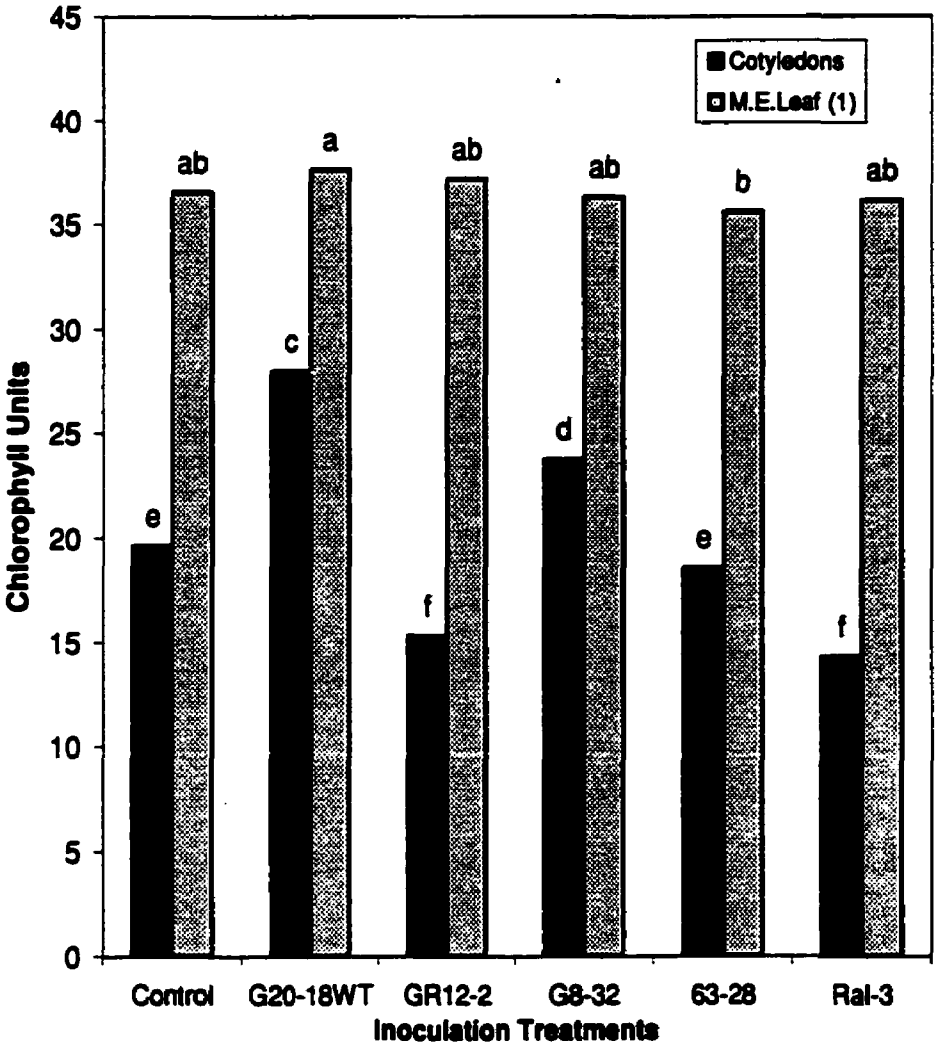


Figure 4.30. Effects of PGPR strains on chlorophyll units present in cotyledons and most expanded leaf of radish plants grown in large pots under greenhouse conditions.

Data are means of 13 pots with three plants. Measurements were done on 20 d-old plants. Same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

(1) M.E. Leaf indicates the most expanded leaf of each plant.

G20-18WT were significantly higher than chlorophyll units measured on plants inoculated with strain 63-28 (P=0.05). The numbers of chlorophyll units in cotyledons were always significantly lower than those for the most expanded leaf 20 d.a.p (P=0.05) (Fig. 4.30). PGPR strains had significantly different effects on the numbers of chlorophyll units in cotyledons of radish plants 20 d.a.p (P=0.05). Plants inoculated with G20-18WT had the highest numbers of chlorophyll units in the cotyledons, followed by strains G8-32 and 63-28. Strains GR12-2 and Ral-3 had the lowest numbers of chlorophyll units in the cotyledons of inoculated plants and these were significantly lower than those of non-inoculated plants (P=0.05).

4.3. Tobacco Callus Bioassay (TCB)

4.3.1. Cytokinin-Dose-Response Experiments

Preliminary experiments were conducted to determine which culture conditions gave the highest sensitivity of tobacco callus to cytokinin addition. Tobacco callus responded differently to increasing concentrations of Z depending on the type of MS24-2 medium used for the TCB and for growing the stock callus. Stock callus grown one step on medium [SD+0] increased fresh weight (FWI) for each level of Z added more than stock tobacco tissues grown on the other three MS24-2 media (Fig 4.31). Significant differences were observed among the control tobacco calli grown on each type of MS24-2 medium (P=0.05). Medium [SD+0] was the most sensitive for use with the TCB (Fig. 4.31). Use of three additional types of stock calli grown two, three or four

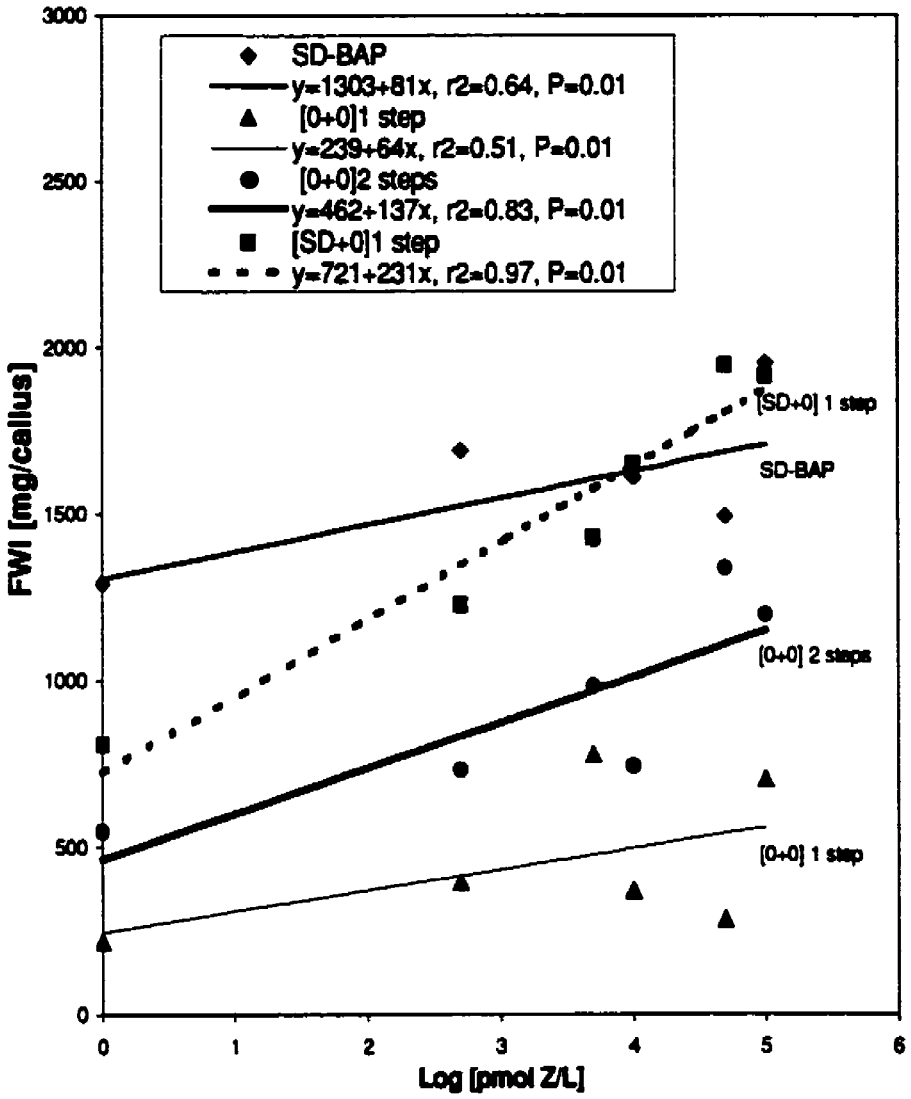


Figure 4.31. Response of tobacco callus to increasing concentrations of Z added to MS24-2 medium, SD-BAP, [0+0] and [SD+0] using stock callus grown on the same type of medium for a variable number of steps.
(1) Stock callus grown at least five steps on SD-BAP medium.
Points are means of a variable number of experiments as described in Table 3.2.

steps on medium [SD+0] did not increase sensitivity to cytokinins (data not shown).

Tobacco callus showed responses to the addition of DHZR but the sensitivity of TCB to this cytokinin was very low. No differences between stock callus and growing media combinations were observed (data not shown).

Data reported in section 4.1 showed that the amounts of ZR (Fig. 4.4) and DHZR (Fig. 4.5) produced by pure cultures of the studied PGPR strains were lower than 500 pmol L^{-1}. Therefore, the response of tobacco callus to the addition of Z concentrations lower than 0.5 n*M* was studied. In addition to the medium combination [SD+0]/[SD+0], two other combinations of stock callus and growing media were included and significant differences among medium combinations were observed in the response of the TCB (Fig. 4.32). The analysis of variance for the linear regression indicated that the variable Log [pmol Z L^{-1}] explained significant amounts of the observed variation in FWI for all the medium combinations ($P<0.001$). The FWI of tobacco callus grown on the control plates of the combination [SD+0]/[SD+0] was significantly higher than those observed for the other combinations ($P=0.05$). However, calli grown on this medium combination showed a lack of sensitivity to Z in the range of 5-500 pmol L^{-1}. In contrast, callus growth on the medium combination [SD+0]/[0+0] had the highest sensitivity to Z in the range of 0-100 pmol L^{-1} and no significant differences were observed for Z concentrations higher than 100 pmol L^{-1}. Linear regression analyses of TCB data obtained for the combinations

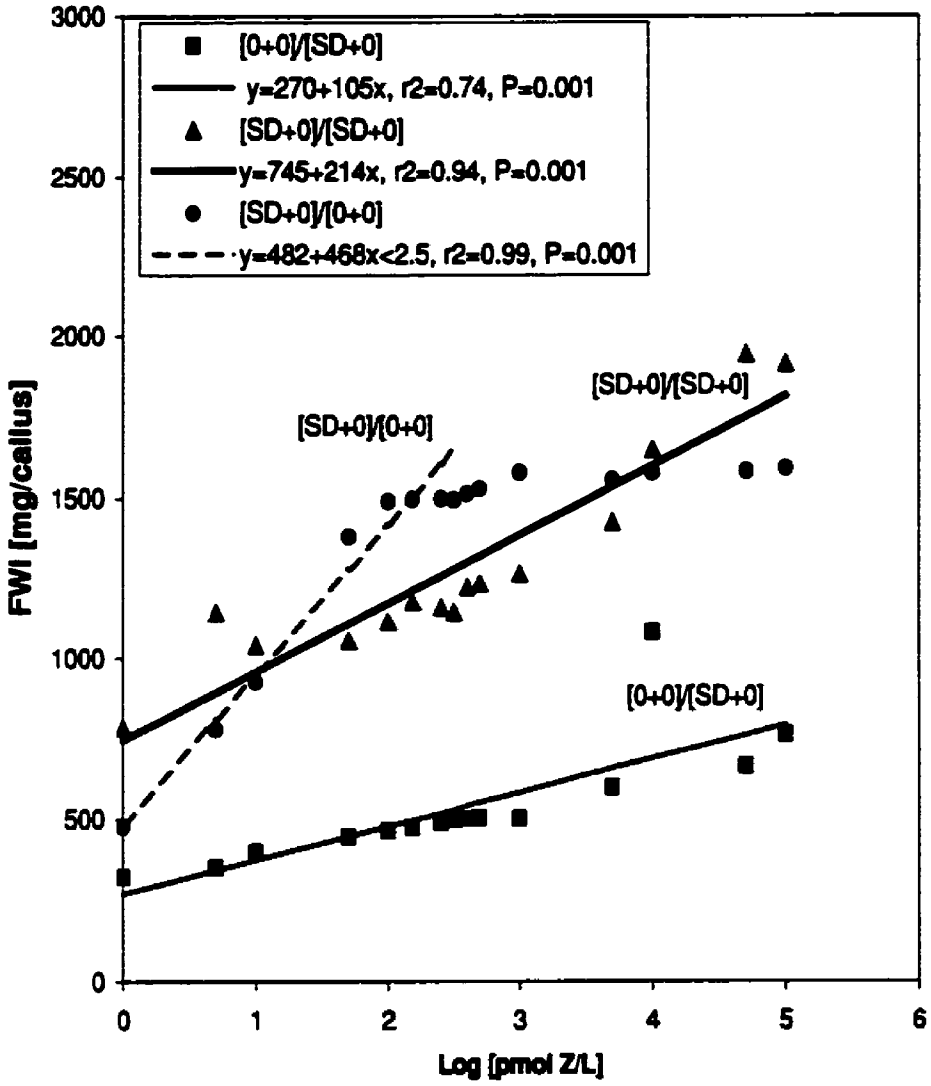


Figure 4.32. Response of tobacco callus to increasing concentrations of Z when grown on three MS24-2 medium combinations.
Points are means of seven experiments.

[0+0]/[SD+0] and [SD+0]/[SD+0] were performed for the full range of Z concentrations.

The standard errors of the constant and the slope for the combination [SD+0]/[SD+0] were the highest (Table 4.31). The standard error of the estimate for the medium combination [SD+0]/[0+0] was the lowest followed by those for the combinations [0+0]/[SD+0] and [SD+0]/[SD+0] in that order.

Table 4.31. Standard errors of constants, regression coefficients (slopes) and estimates of the regression models obtained for the MS24-2 medium combinations used to perform TCB.

Medium Combination [1]	Standard Errors of [2]		
	Constant	Slope	Estimate
[0+0]/[SD+0]	37.3	12.9	118.0
[SD+0]/[SD+0]	89.9	31.1	402.0
[SD+0]/[0+0]	35.3	19.9	82.0

[1] Types of MS24-2 media used to grow the stock callus and to perform the TCB.
[2] The *P* levels for the Student *t* and F tests were lower than 0.001.

The discrepancies between the observed FWI values and the values estimated by the regression models necessitated the inclusion of standard concentrations of cytokinins for each bioassay performed to build the putative cytokinin-dose response line.

Filter sterilization of added Z significantly reduced the variability in the response of the TCB compared to autoclaving Z (Fig. 4.33). For that reason, all the succeeding TCB experiments were conducted using filter-sterilized cytokinins.

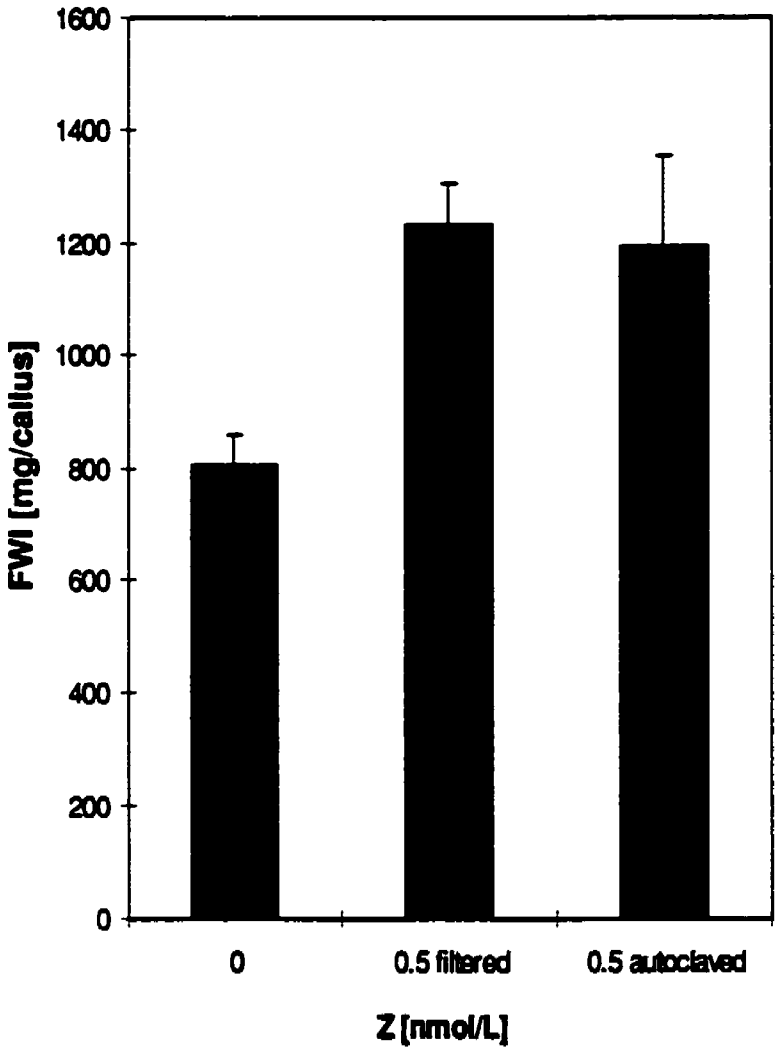


Figure 4.33. Response of tobacco callus to filter sterilized and autoclaved Z added to MS24-2 culture medium.
Bars are means of experiments performed in MS24-2 media as described in Table 3.2 for the indicated treatments. Vertical lines are standard errors of the means.

4.3.2. Time-course of Cytokinin Activity in Supernatants of PGPR Strains

When sterile supernatants of pure cultures of *P. fluorescens* strain G20-18WT grown in MM+Gl medium with and without addition of Ade were added to tobacco callus there were differences in callus growth (Fig. 4.34). Calli grown on control plates always showed a lower FWI than calli grown on plates with addition of supernatants. However, significant differences among treatments were only observed when 96 h-old supernatants were used. The maximum FWI was obtained when 96 h-old supernatants of cultures with addition of 10^{-5} *M* Ade were used and it was significantly higher than FWI obtained with the other treatments. The amounts of zeatin equivalents (ZE) present in plates with addition of 96 h-old supernatants were estimated from the standard curve. Thus, ZE estimated for TCB plates with supernatants of strain G20-18WT with the addition of Ade 10^{-5} *M* were 8.6 fold over those obtained with the addition of Ade 10^{-3} *M*.

Tobacco callus responded differently to the addition of sterile supernatants of strain G20-18WT and its mutants RIF, CNT1 and CNT2 (Fig. 4.35). No significant differences were observed among strains when TCB plates were cultured with supernatants obtained between 4 and 168 h of culture. FWI of calli on plates with 240 and 336 h-old supernatants of transconjugants were significantly lower than those of strains G20-18WT and RIF (P=0.05). FWI of calli on plates with supernatants of strain G20-18WT were always significantly higher than those of strain RIF when both 240 and 336 h-old supernatants were used. The ZE, expressed as pmol L^{-1}, present in the supernatants was estimated

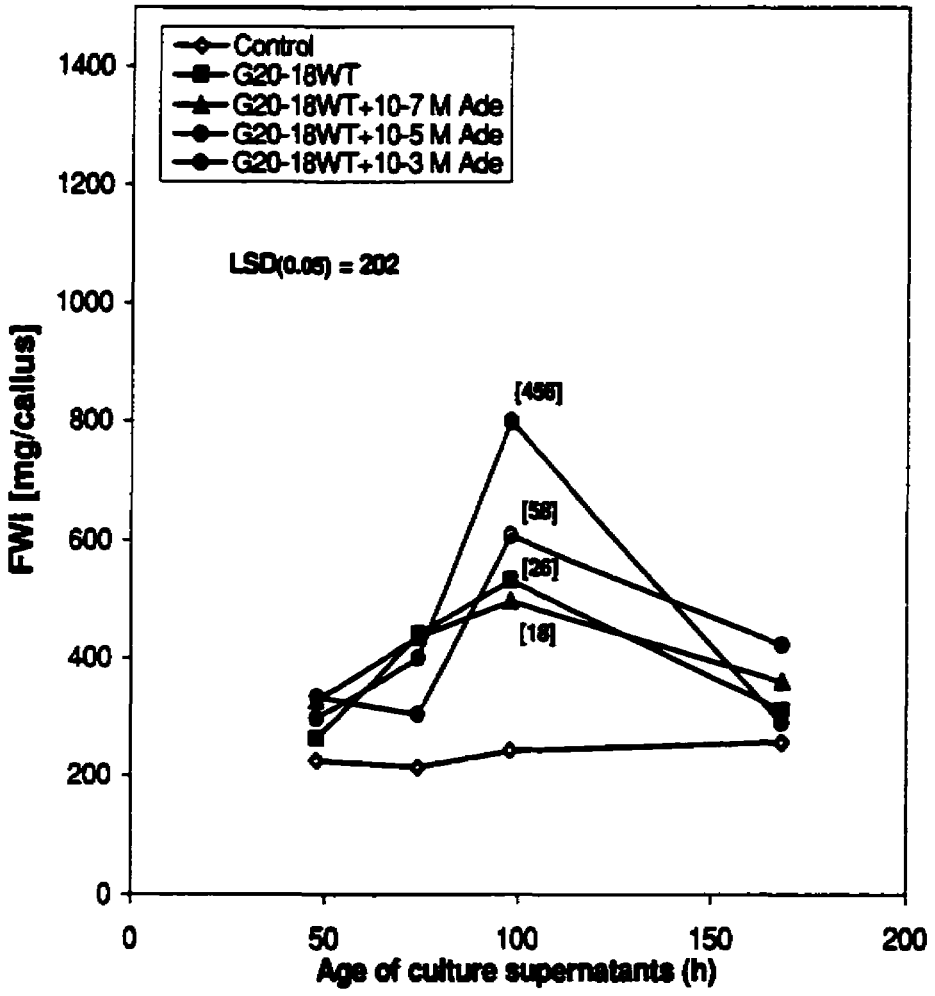


Figure 4.34. Response of tobacco callus to sterile supernatants of *P. fluorescens* strain G20-18WT obtained at different times of culture in MM+Gl with and without addition of adenine.

Data are means of two experiments with three replicate plates. TCB performed on medium [SD+0] using stock callus from one step on medium [SD+0]. Comparison of the means was performed with Tukey's test at the rejection level P=0.05 and the LSD value is indicated in the figure. Numbers in brackets are ZE (pmol L^{-1}) estimated from the equation of the standard line: FWI = 230+214[log (pmol L^{-1})], r^2=0.98, P=0.001.

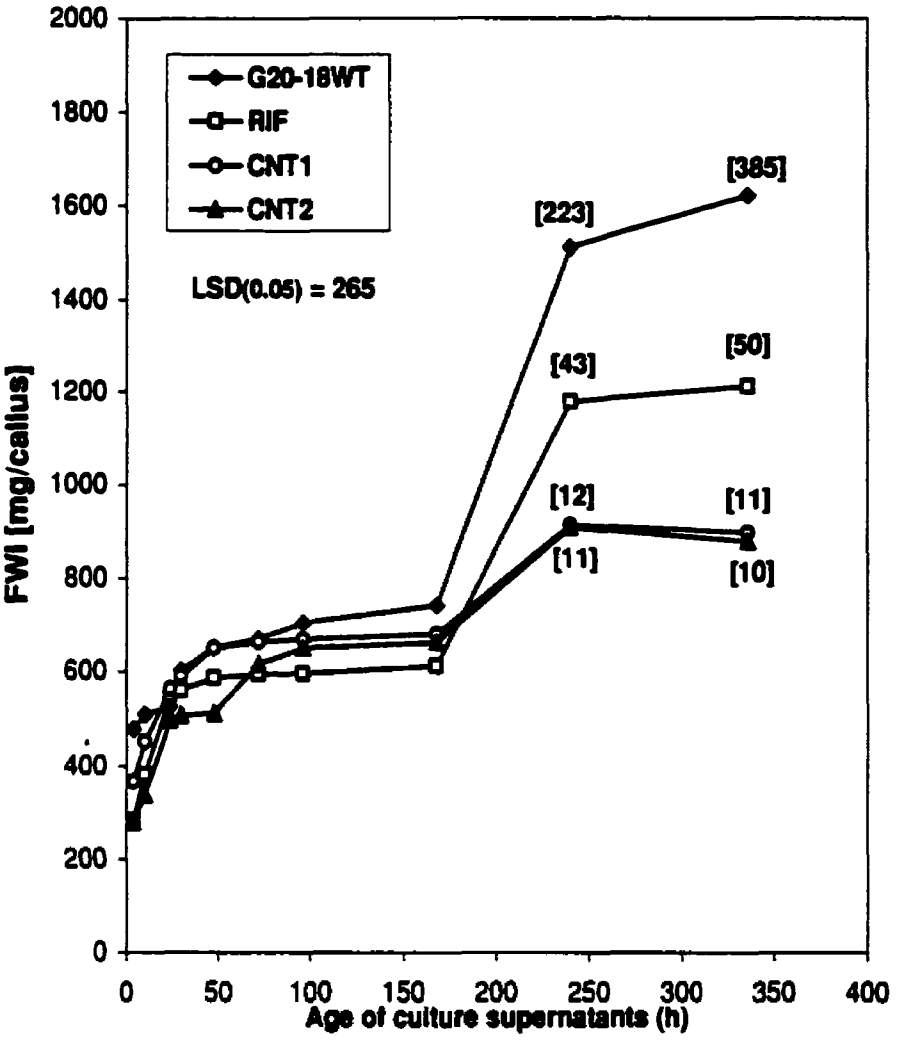


Figure 4.35. Response of tobacco callus to the addition of sterile supernatants of *P. fluorescens* strain G20-18WT and selected mutants into the culture media.

Data are means of two experiments with three replicate plates. TCB performed on medium [0+0] using stock callus from one step on medium [SD+0]. Comparison of the means was performed with Tukey's test at the rejection level P=0.05 and the LSD value is indicated in the figure. Numbers in brackets are ZE (pmol L^{-1}) estimated from the equation of the standard line: FWI = 420+465[log (pmol L^{-1})], r^2=0.99, P=0.001.

from the standard curve. The mean ZE for all the strains in 168-h supernatants was 5 pmol L^{-1}. On average, supernatants of strain G20-18WT older than 240 h had 6.5 and 27.6 fold greater ZE than those of supernatants of the RIF mutant and transconjugants, respectively.

4.3.3. Effects of Selected *Pseudomonas* PGPR Strains on Tobacco Callus Growth.

4.3.3.1. Interaction between medium composition and presence of *P. fluorescens* strain G20-18WT on TCB plates.

The addition of organic substances such as glucose and PAF ingredients (Bacto-tryptone and Bacto-Proteose-3 1:1 w/w) into medium MS24-2 [0+0] and the presence of strain G20-18WT on the TCB plates affected callus growth (Fig. 4.36). Addition of PAF ingredients in concentrations higher than 0.1 g L^{-1} to the medium significantly reduced FWI of callus grown in the inoculated plates by at least 50%. Addition of 1 g L^{-1} of PAF ingredients significantly reduced FWI of callus grown in non-inoculated plates compared to control plates and those with glucose added.

The addition of organic substances and presence of tobacco callus had a significant effect on bacterial survival on TCB plates (Table 4.32). Except for plates with 0.5 and 1 g L^{-1} of PAF ingredients, bacterial numbers after 21 d were significantly lower than those at time zero showing that the survival of strain G20-18WT was negatively affected by the composition of the culture medium. Addition of PAF ingredients in concentrations of 0.5 and 1.0 g L^{-1} significantly

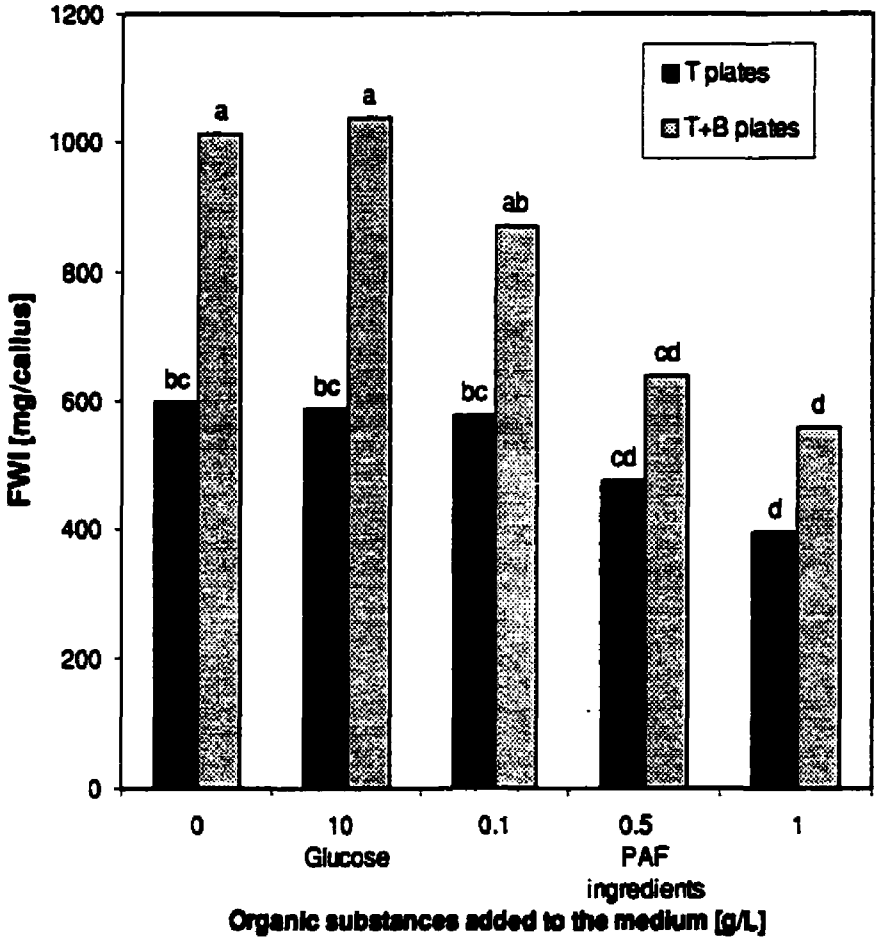


Figure 4.36. Response of tobacco callus to the presence of *P. fluorescens* strain G20-18WT and addition of organic substances to the culture medium MS24-2 [0+0].

Bars are means of three experiments with three replicate plates. Stock callus grown one step on medium [SD+0]. Calluses were harvested after 21 days. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. T and T+B: Indicate TCB plates with tobacco callus only and both tobacco callus tissues and bacterial cultures, respectively.

Table 4.32. Effect of medium composition on the survival of *P. fluorescens* strain G20-18WT after culturing on TCB plates for 7, 14 and 21 d with and without tobacco callus.

Organic substances added to the medium MS24-2 [0+0] [g L^{-1}]	Bacterial Numbers Log [cfu $plate^{-1}$]						
	Time 0 (3)	7 d		14 d		21 d	
		B (4)	T+B (4)	B	T+B	B	T+B
0	9.6	6.6	7.7	6.2	7.3	5.1	7.9
Glucose							
10	9.6	6.8	7.7	6.3	7.7	5.9	7.2
PAF ingredients (1)							
0.02	10.1	7.4	7.5	7.1	7.5	6.4	7.2
0.05	9.6	7.4	7.4	6.9	7.1	6.2	6.2
0.1	9.7	nd (5)	nd	nd	nd	6.0	7.3
0.5	9.7	nd	nd	nd	nd	11.1	13.0
1.0	9.7	nd	nd	nd	nd	13.5	13.6
LSD (2) (P=0.05)	0.7						

Data are means of three experiments with three replicate plates. TCB performed using stock callus grown one step on medium [SD+0].

(1) PAF ingredients are Bacto-tryptone and Bacto-Proteose-3 added 1:1 w/w.

(2) LSD is the critical value for the comparison of the means performed with Tukey's test at the rejection level P=0.05.

(3) Bacterial counts performed after 72 h of incubation at 30°C and before placing the pieces of callus on the plates. Initial suspensions of all strains contained Log 9.3 cfu plate-1.

(4) B and T+B: Indicate TCB plates with bacterial cultures only and both tobacco callus and bacterial cultures, respectively.

(5) nd: not determined

increased the population of strain G20-18WT on the plates. In most cases, the presence of tobacco callus significantly increased the survival of strain G20-18WT, but no significant differences were observed among these three treatments. Bacterial counts performed at 7, 14 and 21 d showed significant interactions between the addition of organic substances and the presence of

tobacco callus. After 7 d, all treatments had significantly lower bacterial numbers than those estimated at time zero. The presence of tobacco callus in control plates and those containing glucose had a significant positive effect on the survival of strain G20-18WT and the bacterial numbers were significantly higher than those estimated on B plates at 7, 14 and 21 d (P=0.05). Bacterial numbers obtained for B plates of the former two treatments at 7 d were significantly higher than those obtained at 21 d.

At 21 d, bacterial numbers estimated for T+B plates with glucose were similar to those estimated for T+B control plates but B plates with glucose had significantly higher bacterial numbers than B control plates.

In summary, addition of PAF ingredients in the concentrations tested increased bacterial survival but reduced the growth of tobacco callus. When PAF ingredient concentrations were reduced to 0.02 and 0.05 g L^{-1} callus growth was increased relative to the control, but there were no differences among treatments in the presence of G20-18WT (Fig. 4.37). A time course assay in the absence of glucose or PAF ingredient additions indicated significant differences between inoculation treatments when the tobacco callus was harvested at 7, 14 and 21 d (P=0.001), (Fig. 4.38). On average strain G20-18WT produced 204±10 pmol L^{-1} of ZE, which was calculated from the FWI of calli harvested after 21 d (Fig. 4.38) as estimated from the indicated standard curve (Fig. 4.35).

From these data it was concluded that medium [0+0] had the highest sensitivity with enough living bacterial cells at the end of the TCB to study the production of cytokinins by PGPR strains in the presence of tobacco callus.

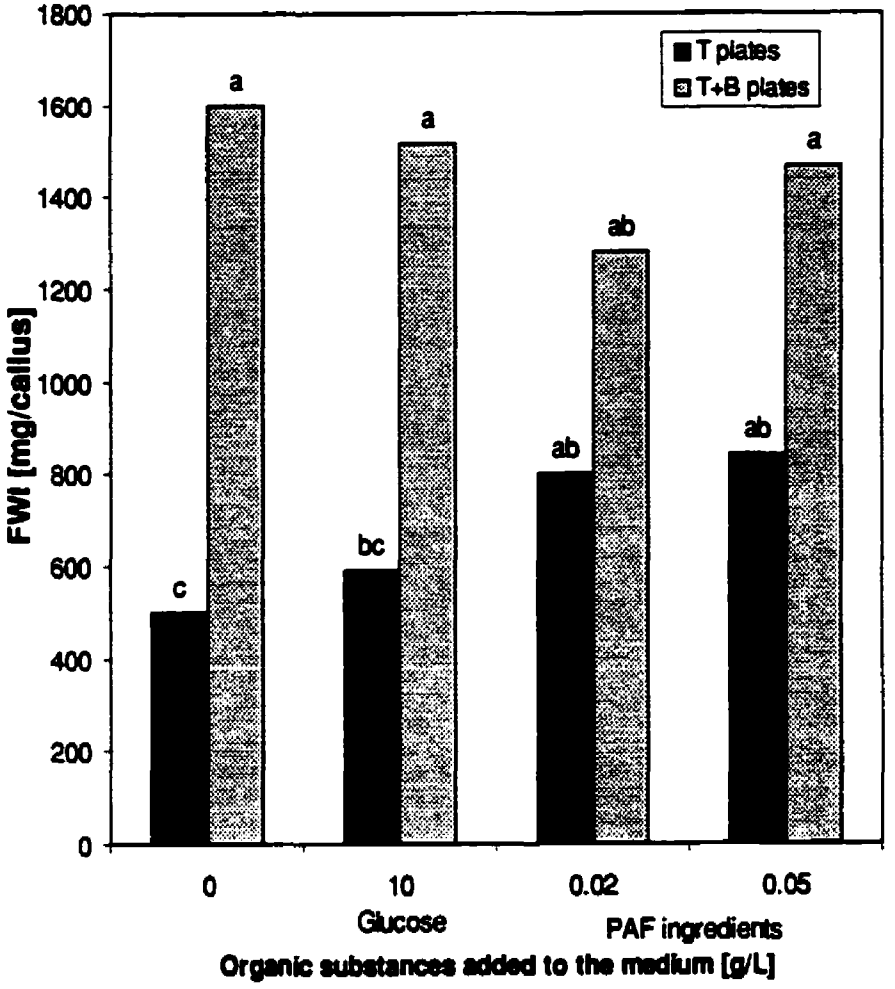


Figure 4.37. Response of tobacco callus to the presence of *P. fluorescens* strain G20-18WT and addition of glucose and two concentrations of PAF ingredients to the culture plates after 21 days of growth.
Bars are means of three experiments with three replicate plates. TCB performed on MS24-2 [0+0] using stock callus grown one step on medium [SD+0]. Bars with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05. T and T+B: Indicate TCB plates with tobacco callus only and both tobacco callus and bacterial cultures, respectively.

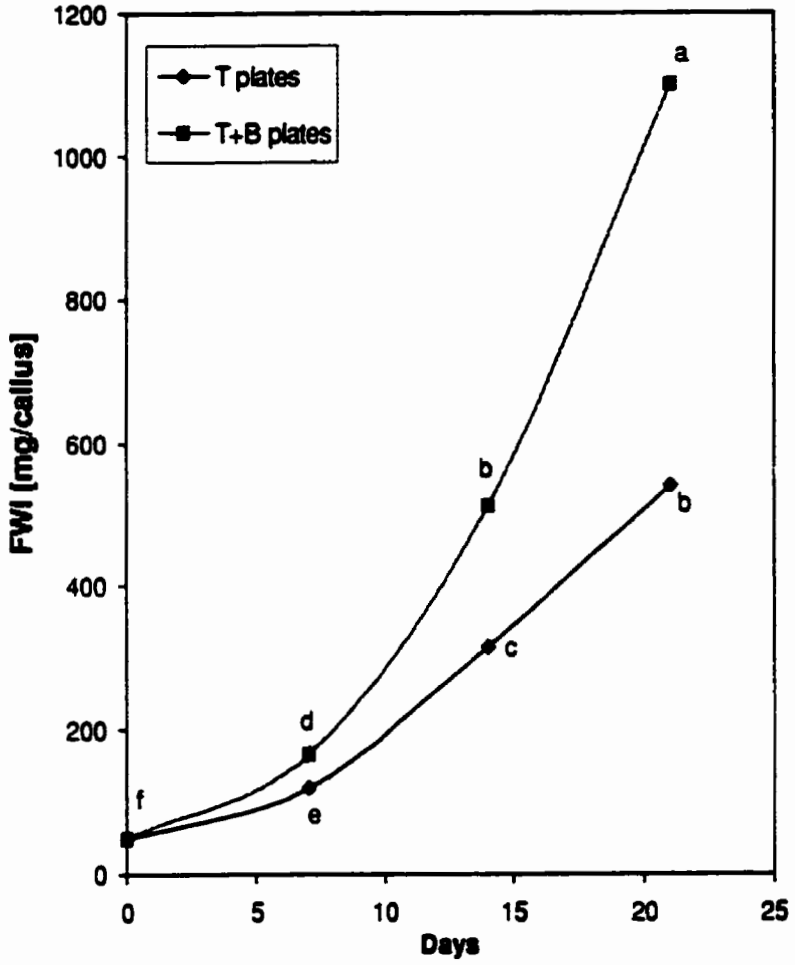


Figure 4.38. Time course of the response of tobacco callus tissues to the presence of *P. fluorescens* strain G20-18WT in the TCB culture plates.
Data are means of 8 experiments performed on medium [0+0] using stock callus grown one step on medium [SD+0]. Means with the same letter indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.001. T and T+B: Indicate TCB plates with tobacco callus only and both tobacco callus tissues and bacterial cultures, respectively.

4.3.3.2. Detection of cytokinin-like compounds produced by certain *Pseudomonas* PGPR strains in the presence of tobacco callus.

The fresh weight of tobacco callus grown one step on medium [SD+0] significantly increased when cultured on media [SD+0] and [0+0] in the presence of strains G20-18WT, G8-32 and 63-28 (Tables 4.33 and 4.34). However no differences among strains were observed. ZE expressed as pmol L^{-1} was calculated from the FWI data using the standard curve, but there were no differences among strains on medium [SD+0] (Table 4.33). On medium [0+0] strain G20-18WT produced significantly more ZE than strain 63-28 (P=0.05) (Table 4.34).

Table 4.33. Response of tobacco callus to the presence of three PGPR strains cultured in the same plates on medium [SD+0].

Strains	FWI [1a] (mg callus^{-1})	ZE [2] (pmol L^{-1})	Bacterial Numbers [3] Log (cfu plate^{-1})	ZE-cfu [4] nmol (10^{10} cfu)$^{-1}$
Control	512 b[1b]	-	-	-
G20-18WT	1175 a	127 a	7.2 a	20.3 a
G8-32	1269 a	235 a	7.4 a	26.3 a
63-28	1230 a	182 a	8.1 a	3.6 b
P	0.05	0.05	0.05	0.01

Data are means of three experiments with three replicate plates. TCB performed using stock callus grown one step on medium [SD+0]. Calluses were harvested after 21 d.

[1a] FWI indicates fresh weight increase of tobacco callus.

[1b] Means followed by the same letter are similar as determined by Tukey's test at the indicated rejection level *P*.

[2] ZE indicates Z equivalents, estimated from the standard line equation, FWI=432+352[log(pmol Z L^{-1})]; r^2=0.91, P=0.01.

[3] Bacterial numbers counted after 7 d of incubation at 30°C and before placing the pieces of callus on the plates.

[4] ZE-cfu indicates Z equivalents expressed as nmol per 10^{10} cfu.

Table 4.34. Response of tobacco callus to the presence of three PGPR strains cultured in the same plates on medium [0+0].

Strains	FWI [1] (mg callus^{-1})	ZE [2] (pmol L^{-1})	Bacterial Numbers [3] Log (cfu plate^{-1})	ZE-cfu [4] nmol (10^{10} cfu^{-1})
Control	537 b[5]	-	-	-
G20-18WT	1458 a	205.6 a	7.2 a	32.20 a
G8-32	1390 a	142.2 ab	7.4 a	15.88 b
63-28	1266 a	72.6 b	8.1 a	1.44 c
P	0.05	0.05	0.05	0.01

Data are means of four experiments with three replicate plates. TCB performed using stock callus grown one step on medium [SD+0]. Calluses were harvested after 21d.
[1] FWI indicates fresh weight increase of tobacco callus.
[2] ZE indicates Z equivalents, estimated from the standard line equation, FWI=475+425[log(pmol Z L^{-1})]; r^2=0.93, *P*=0.01.
[3] Bacterial numbers counted after 7 d of incubation at 30°C and before placing the pieces of callus on the plates.
[4] ZE-cfu indicates Z equivalents expressed as nmol per 10^{10} cfu.
[5] Means followed by the same letter are similar as determined by Tukey's test at the indicated rejection level *P*.

On medium [SD+0], the ZE-cfu produced by strains G20-18WT and G8-32 was similar and 5.6 and 7.3 fold greater than that produced by strain 63-28, respectively (Table 4.33). On medium [0+0], the ZE-cfu produced by strain G20-18WT was significantly higher than those by strain G8-32 and strain 63-28 (Table 4.34).

These results show that selected PGPR strains produce cytokinin-like compounds in the presence of plant tissues, such as tobacco callus cultured on media [SD+0] and [0+0]. However assays performed on the latter medium were more sensitive to differences among strains when the ZE was expressed as pmol L^{-1}. In all cases, the amounts of ZE were lower than 500 pmol L^{-1} and the estimates have an acceptable level of accuracy because standard concentration

points lower than 500 pmol L^{-1} of Z were included in all the TCB experiments in order to obtain the standard curve. Also it was observed that the biggest differences among strains were detected when the ZE produced by them were expressed as nmol $(10^{10}$ cfu$)^{-1}$ for both types of media.

4.3.3.3. Effect of *P. fluorescens* strain G20-18WT and selected mutants on tobacco callus growth and cytokinin production.

The fresh weight of tobacco callus cultured on medium [0+0] increased significantly after 14 d when PGPR strains were present (P=0.05) (Fig. 4.39). However, significant differences among strains were detected after 21 d when the fresh weight increases of callus cultured in the presence of strains G20-18WT and RIF were similar and approximately 2.1 fold higher than those cultured in the presence of strains CNT1 and CNT2 (P=0.05).

Bacterial counts performed at 7, 14 and 21 d showed significant differences among PGPR strains and in the presence of tobacco callus (Table 4.35). All plates had similar bacterial numbers when the TCB began. After 7 d, B and T+B plates had significantly lower bacterial numbers than those estimated at time zero (P=0.05). After 14 d, the numbers of strains G20-18WT and RIF on B and T+B plates were similar to each other and significantly less than those at 7 d (P=0.05). They were also significantly lower than those for mutants CNT1 and CNT2 on both B and T+B plates. All inoculated plates had countable cells after 21 d but the numbers of all strains on B plates were significantly reduced and

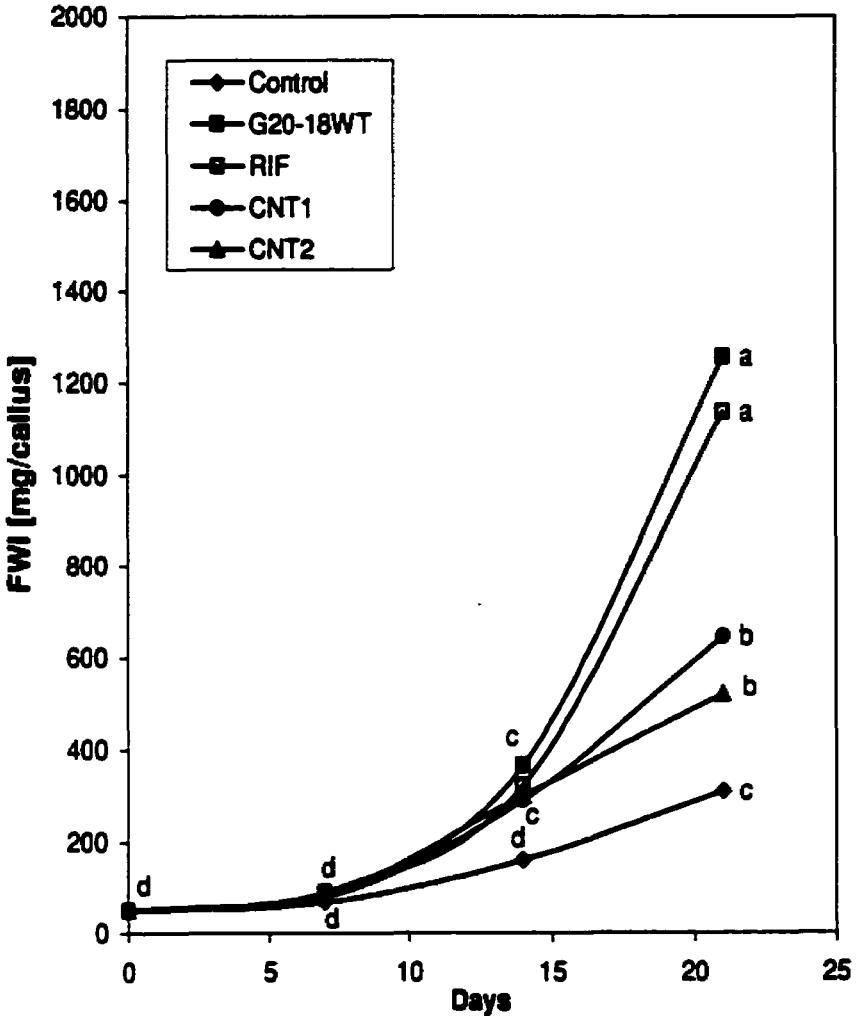


Figure 4.39. Response of tobacco callus to the presence of P. fluorescens strain G20-18WT and three selected mutants on TCB culture plates.
Data are means of three experiments performed on medium [0+0] using stock callus grown one step on medium [SD+0]. Means with the same letter indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

lower than those on T+B plates. Numbers of strains G20-18WT and RIF on T+B plates after 14 and 21 d were similar but numbers of mutants CNT1 and CNT2 on T+B plates were significantly reduced after 21 d.

Table 4.35. Numbers of *P. fluorescens* strain G20-18WT and selected mutants on TCB plates cultured for 7, 14, and 21 d with and without tobacco callus.

PGPR Strains	Bacterial Numbers Log [cfu plate^{-1}]						
	Time 0	7 d		14 d		21 d	
	[2]	B [3]	T+B [3]	B	T+B	B	T+B
G20-18WT	9.7	7.7	7.9	4.7	5.8	3.1	5.9
RIF	9.3	7.4	7.5	4.2	5.6	2.7	5.5
CNT1	10.3	8.1	8.3	7.0	8.3	2.7	5.5
CNT2	9.9	8.0	8.2	6.1	8.8	2.5	5.9
LSD [1] (P=0.05)	1.5						

Data are means of three experiments with three replicate plates. TCB performed on medium [0+0] using stock callus grown one step on medium [SD+0].
[1] LSD is the critical value for the comparison of the means performed with Tukey's test at the rejection level P=0.05.
[2] Bacterial counts performed after 72 h of incubation at 30°C and before placing the pieces of callus on the plates. Initial suspensions of all strains contained Log 9.3 cfu plate^{-1}.
[3] B and T+B: Indicate TCB plates with bacterial cultures only and both tobacco callus and bacterial cultures, respectively.

The Z equivalents produced by strain G20-18WT and the three mutants were calculated from the FWI of calli harvested at 21 d (Fig. 4.39) using the standard curve (Table 4.36). Strains G20-18WT and RIF produced similar amounts of ZE. The transconjugants produced significantly lower amounts of ZE than strain G20-18WT. The amounts of ZE-cfu produced by the transconjugants were significantly lower than those produced by G20-18WT and mutant RIF.

Table 4.36. ZE produced by *P. fluorescens* strain G20-18WT and three selected mutants after 21 d of growth on T+B plates with medium [0+0].

Strains	ZE [1] (pmol L^{-1})	ZE-cfu [3] [nmol (10^{10} cfu)$^{-1}$]
G20-18WT	212.6 a [2]	50.6 a
RIF	84.1 ab	39.9 a
CNT1	3.7 b	1.0 b
CNT2	1.5 b	0.2 b

[1] ZE, indicates Z equivalents, estimated from the standard line equation, FWI=459+342[log(pmol Z L^{-1})]; r^2=0.97, P=0.01.
[2] Means followed by the same letter are similar as determined by Tukey's test at the indicated rejection level P.
[3] ZE-cfu indicates Z equivalents per 10^{10} cfu estimated using a weighted average of bacterial numbers of each strain counted at 7, 14 and 21 d.

The amounts of IPA, ZR and DHZR present in medium [0+0] after 7, 14 and 21 d differed among TCB treatments (Tables 4.37, 4.38 and 4.39). The total amounts of cytokinins [IPA+ZR+DHZR] determined by immunoassay were higher than those estimated from the regression line obtained from Z-dose response experiments (Table 4.36). Regardless of the TCB treatments, the amounts of IPA in the plates increased during the bioassays (Table 4.37). The comparison of means was performed for every sampling date because no interaction was observed between sampling dates and TCB treatments. After 7 d, pure cultures of all the strains produced significantly higher amounts of IPA than strains cultured with tobacco callus. Regardless of the presence of tobacco callus, TCB plates of strains G20-18WT and RIF had significantly higher amounts of IPA than those estimated for plates with mutants CNT1 and CNT2 after 21 d (P=0.05). After 7 d, T plates had significantly higher amounts of IPA

than plates with tobacco and bacteria (T+B plates) (P=0.05). After 14 and 21 d, the amounts of IPA in T plates were similar to those estimated for T+G20-18WT plates.

Table 4.37. IPA production by *P. fluorescens* strain G20-18WT and three selected mutants cultured alone or in the presence of tobacco callus on TCB plates containing medium [0+0].

TCB Plates [1]	IPA production after days of tobacco callus growth [nmol plate^{-1}]		
	7	14	21
G20-18WT	30.4 a	62.0 a	1524.3 ab
RIF	26.8 a	25.9 bcd	1346.1 ab
CNT 1	11.8 b	20.2 bcd	185.3 d
CNT 2	11.4 b	19.9 bcd	152.9 d
T	7.9 b	14.7 d	1348.1 ab
T+G20-18WT	6.1 x 10^{-2} c	16.5 cd	2091.0 a
T+RIF	6.1 x 10^{-2} c	37.9 b	1008.3 bc
T+CNT 1	1.0 x 10^{-2} c	35.3 b	146.3 d
T+CNT 2	0.9 x 10^{-2} c	32.5 b	143.2 d

[1] G20-18WT, RIF, CNT1 and CNT2: indicate TCB plates with pure cultures of the respective PGPR strain without tobacco callus. T: indicates TCB plates with tobacco callus only, T+WT, T+RIF, T+CNT1 and T+CNT2: indicate TCB plates with tobacco callus and the respective PGPR strain.
Data are means of three experiments with three replicate plates. Means with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05 for every sampling date.

After 14 d, strains G20-18WT and RIF produced more ZR than at 7 and 21 d in B plates, but strain RIF produced significantly less ZR than strain G20-18WT (P=0.05), (Table 4.38). However, B plates of strain G20-18WT had significantly higher amounts of ZR than T+B plates of this strain. Pure culture plates of all the strains had similar amounts of ZR at 7 and 21 d. The amounts of ZR in T+B plates of the three mutants at 7 and 14 d were similar and significantly lower

than those estimated after 21 d (P=0.05). T+B plates of mutants RIF and CNT2 had significantly higher amounts of ZR than the pure culture plates after 21 d (P=0.05). In contrast, the amounts of ZR in pure culture and T+B plates of mutant CNT1 were similar to each other.

Table 4.38. ZR production by *P. fluorescens* strain G20-18WT and three selected mutants cultured alone or in the presence of tobacco callus on TCB plates containing medium [0+0].

TCB Plates [(1)]	ZR production after days of tobacco callus growth [nmol plate^{-1}]		
	7	14	21
G20-18WT	8.0 def	2168.5 a	17.9 def
RIF	1.1 f	52.8 c	15.2 def
CNT 1	5.0 def	29.6 cdef	32.2 cde
CNT 2	4.0 ef	23.3 cdef	36.2 cde
T	0.4 f	38.9 cd	0.4 f
T+G20-18WT	3.2 x 10^{-5} f	32.9 cde	5.5 def
T+RIF	3.7 x 10^{-5} f	0.2 f	138.8 bc
T+CNT 1	1.7 x 10^{-5} f	0.2 f	186.3 bc
T+CNT 2	3.1 x 10^{-5} f	0.2 f	350.7 b

[(1)] G20-18WT, RIF, CNT1 and CNT2: indicate TCB plates with pure cultures of the respective PGPR strain without tobacco callus. T: indicates TCB plates with tobacco callus only, T+WT, T+RIF, T+CNT1 and T+CNT2: indicate TCB plates with tobacco callus and the respective PGPR strain.
Data are means of three experiments with three replicate plates. Means with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

The amounts of DHZR produced by strain G20-18WT were significantly higher than those produced by the three selected mutants in both pure culture and in the presence of tobacco callus, except in pure culture after 21 d (Table 4.39). G20-18WT produced 90, 98 and 47 fold more DHZR in the presence of

tobacco callus than in pure culture at 7, 14 and 21 d, respectively. In contrast, the mutants produced similar or more DHZR in pure culture than in the presence of tobacco callus.

Table 4.39. DHZR production by *P. fluorescens* strain G20-18WT and three selected mutants cultured alone or in the presence of tobacco callus on TCB plates containing medium [0+0].

TCB Plates [1]	DHZR production after days of tobacco callus growth [nmol plate^{-1}]		
	7	14	21
G20-18WT	293.34 ef	437.79 d	65.80 ghi
RIF	63.29 ghi	115.02 ghi	84.82 ghi
CNT 1	139.23 gh	167.20 fg	42.95 ghi
CNT 2	84.49 ghi	98.57 ghi	50.18 ghi
T	62.50 ghi	107.53 ghi	368.51de
T+G20-18WT	5,627 c	10,540 b	18,270 a
T+RIF	2.74 i	3.08 i	125.89 ghi
T+CNT 1	0.17 i	1.38 i	22.29 hi
T+CNT 2	0.02 i	0.26 i	80.98 ghi

[1] G20-18WT, RIF, CNT1 and CNT2: indicate TCB plates with pure cultures of the respective PGPR strain without tobacco callus. T: indicates TCB plates with tobacco callus only, T+WT, T+RIF, T+CNT1 and T+CNT2: indicate TCB plates with tobacco callus and the respective PGPR strain.
Data are means of three experiments with three replicate plates. Means with the same letter/s indicate no significant differences between means as determined by Tukey's test at the rejection level P=0.05.

Although TLC chromatograms of ethyl acetate fractions of TCB agar samples showed no visible spots at the R_F section of IAA, agar extracts were analyzed immunologically for IAA-like substances. There were no differences among strains after 21 d. There was no interaction between PGPR strains and the presence or absence of tobacco callus. However, the mean concentration of

IAA in plates with tobacco alone was significantly lower than those in plates with bacteria alone or bacteria plus tobacco callus (Table 4.40).

Table 4.40. Average concentrations of IAA present in TCB plates containing medium [0+0] after 21 d.

Type of TCB plates [1]	IAA (nmol plate^{-1})
T	424.2 b
B	4441.8 a
T+B	5157.6 a

Data are means of three experiments performed on medium [0+0] using stock callus grown one step on medium [SD+0].
[1] The comparison of means was performed among types of TCB plates because the analysis of variance showed neither interaction nor differences among strains. Means with the same letter are similar as determined by Tukey's test at the rejection level P=0.05.

There were no differences among strains in lettuce hypocotyl bioassays performed for two R_F sections of TLC chromatograms of ethyl acetate fractions of TCB agar samples after 21 d. Hypocotyl elongation values ranged between 1.03 and 2.63 cm plant^{-1}, for the water control and the control mixture solution containing 10 mM of GA_3, respectively (Fig. 4.40). No significant interaction was observed between PGPR strains and types of TCB plates. The average concentration of gibberellin-like substances in the T+B plates was 0.016 mg L^{-1} of GA_3 equivalents as determined from the dose-response line described in Appendix B. Hypocotyl elongation values obtained with the section of R_F=0.0-0.2 were similar to the water control values for all TCB treatments.

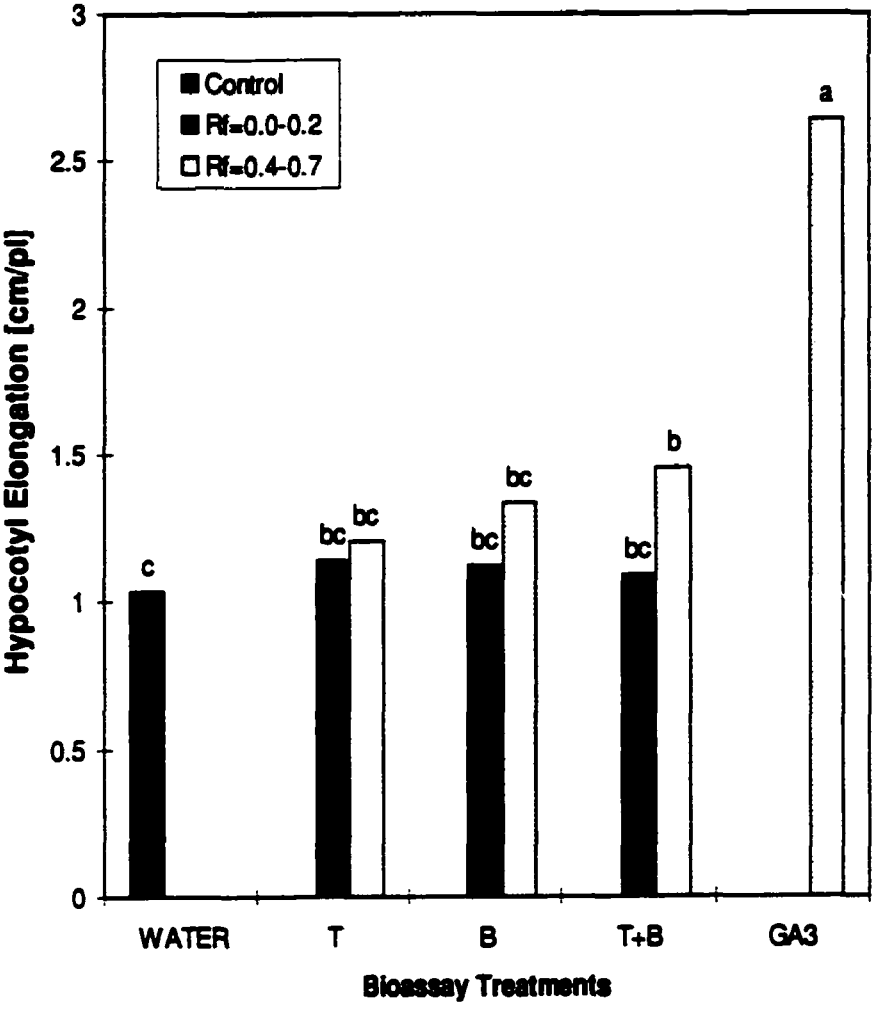


Figure 4.40. Effect of water and TLC eluted samples of ethyl acetate fractions of gibberellic acid control solution and agar extracts of TCB plates incubated for 21 d.

Bars are means of three bioassays. TLC was performed in solvent chloroform:ethyl acetate:formic acid (50:20:10, v/v) using 20 µL for sample. Spots at indicated R_F sections were eluted, dried as described in section 3.4.2. Water solutions were prepared and the bioassay was performed as described in Appendix 2. TLC spots of authentic GA_3 present in co-chromatographed extracts of control mixture solution containing 10 m*M* of GA_3 were also eluted and included in the bioassays. Means with the same letter are similar as determined by Tukey's test at the rejection level P=0.05. T, B and T+B: Indicate TCB plates with tobacco callus tissues only, bacterial cultures only and both tobacco callus tissues and bacterial cultures, respectively.

CHAPTER 5. DISCUSSION

5.1. Cultures of selected PGPR strains.

Although production of PGRs has been one of the mechanisms used to explain direct effects of PGPR on plant growth, most of the studies have been concerned with the role of auxin-producing PGPR (Glick 1995). As cytokinin-producing PGPR have been found naturally, they represent an alternative mechanism by which PGPR exert their beneficial effects on plant tissues.

DHZR production in pure cultures varied among the PGPR strains studied (Table 4.1) and it was lower than those reported for *Azotobacter chroococcum* strain ATCC 9043 (Nieto and Frankenberger 1989) and a *P. fluorescens* strain isolated from the rhizosphere of maize (Muller et al. 1988) after 72 h of growth.

B. cepacia strain Ral-3 has been reported to be a biological control agent of soil-borne pathogens with the most consistent performance across multiple crops among 500 strains (Pedersen and Reddy 1996). Although this strain has potential use in the forestry industry (Reddy et al. 1997), the mechanism(s) of biological control or growth promotion have not been elucidated. Preliminary investigations indicated that strains Ral-3 and *P. chlororaphis* 63-28 produced anti-fungal volatiles, siderophores, chitinases, phosphatases and IAA (Pastula et

al. 1996). The results reported in this thesis suggest that cytokinin production by Ral-3 is not responsible for the positive effects on plant growth observed in the absence of pathogens.

Strain 63-28 has also been investigated as a potential biological agent to protect and improve the yield of diverse horticultural and floricultural crops in greenhouses (Reddy et al. 1990; Gagne et al. 1993). Garnard et al. (1996, 1997) reported that this strain produced a minimum of four different anti-fungal metabolites. Ultra-structural observations showed that strain 63-28 functions as an inducer of plant disease resistance by increasing callose depositions and creating a fungi-toxic environment (Piga et al. 1997). Inoculation experiments performed with strain 63-28 and *P. putida* strain G8-32 showed that these strains could increase root growth of canola, cucumber and tomato in laboratory conditions in the absence of pathogens (Reddy et al. 1990). These authors reported higher production of DHZR and ZR by these two strains than those reported here for bacterial supernatants (Table 4.1) and in the presence of tobacco callus tissues (Tables 4.38 and 4.39). Although both the present study and that by Reddy et al. (1990) used immunoassays, the differences could be due to different culture conditions (Nieto and Frankenberger 1989) and to the characteristics of the ELISA kit. In the latter study the commercial kits used were undergoing standardization (G. Brown, personal communication).

Recently, it was demonstrated that *P. putida* strain GR12-2 contains ACC deaminase and promotes growth of canola by lowering the endogenous ethylene concentration of the plant (Glick 1995), but the ability to produce cytokinins has

not been tested before. My work showed that strains GR12-2 and 63-28 have the ability to produce similar amounts of DHZR in MM+Gl medium.

Production of DHZR was a good screening tool to identify Tn5-insertion mutants of strain G20-18WT with impaired ability to produce cytokinins (Fig. 4.1). Although spontaneous antibiotic resistance has provided a potentially simple and effective method to mark bacterial strains, the reduced ability of mutant RIF to produce IPA (Fig. 4.3), ZR (Fig. 4.4) and DHZR (Fig. 4.5) compared to the wild-type strain may be related to a genetic lesion induced by the antibiotic (Bromfield et al. 1985). Compeau et al. (1988) classified several *P. fluorescens* rifampicin-resistant mutants and concluded that mutants having similar growth rates in the culture medium to the wild type also had similar competitive fitness. In this study, strain G20-18WT and the RIF mutant had similar growth rates in MM+Gl medium (Fig. 4.2) and colonization abilities (Table 4.9, Fig. 4.28).

The three cytokinins measured throughout this study are strategically synthesized in the general metabolic pathway (Fig. 2.3). IPA is the second metabolite formed and two enzymatic reactions are involved in its synthesis, which seemed to be greatly affected in the transconjugants when they were in the stationary phase of growth (Fig. 4.2 and 4.3). The amount of ZR and DHZR estimated at any particular time of incubation is a function of the amount of IPA produced previously.

In the general metabolic pathway described in Fig. 2.3, ZR is an intermediate metabolite, which can be synthesized from IPA in a one-step

reaction catalyzed by oxidase enzymes and also in several enzymatic steps involving successive nucleotidases, mixed oxidases and Ade phosphorylase enzymes to produce the metabolites IPa and Z. Although the specific enzymes are unknown, and were not studied in this work, these data showed that the activity of the enzymes involved in the synthesis of ZR was severely affected in the transconjugants.

As described by several authors, DHZR is one of the final derivatives in the metabolic pathway (Fig. 2.3) synthesized without direct connection between IPA and ZR. Z is an intermediate metabolite between ZR and DHZR and several enzymes can regulate the formation of an array of different metabolites (Kaminek 1992; Jameson 1994; Letham and Palni 1983). The Z-reductase catalyzing the synthesis of DHZ and the Ade phosphorylase catalyzing the synthesis of ZR compete for the same substrate (Fig. 2.3). Although the synthesis of DHZR requires an additional reaction catalyzed by an Ade phosphorylase, the data shown in Figs. 4.4 and 4.5 indicate that most of the Z produced by strain G20-18WT and its selected mutants is metabolized by the Z-reductase to synthesize the precursor DHZ. As this metabolite was not detected in the TLC chromatograms of bacterial supernatant extracts, it can be assumed that it is quickly metabolized to produce DHZR in a reaction catalyzed by a phosphorylase enzyme.

Although, no transconjugants were found with completely suppressed cytokinin metabolism, several enzymatic steps of the cytokinin biosynthetic pathway appeared to be partially suppressed in the selected Tn5-insertion

mutants (CNT1 and CNT2). The increasing percentages of ZR and DHZR accumulated by the transconjugants after 240 h of culture indicated that these mutants were not producing new IPA as its percentage decreased (Fig. 4.3 and Table 4.3). The former two metabolites were probably synthesized from IPA present in the medium. In contrast strain G20-18WT continued to produce IPA and as a result the actual amounts of ZR and DHZR produced by this strain were higher than those produced by the transconjugants.

The observed changes in cytokinin metabolism of the transconjugants could be related to the observed differences between strain G20-18WT and mutants in their patterns of C-source utilization (Fig. 4.7). Several authors have suggested that differences in C-source utilization could change the way mutants establish an association with plant roots or tissues (O'Sullivan and Stephens 1991; Fisher and Long 1992). However, while mutants CNT1 and CNT2 and strain G20-18WT colonized wheat (Table 4.9) and radish (Fig. 4.28) in a similar way and their numbers in TCB plates after 21 d (Table 4.35) were similar, the mutants did not increase plant growth. They grew similarly to strain G20-18WT in MM+Gl medium and showed similar generation times (Fig. 4.2), but produced significantly lower amounts of cytokinins in both pure cultures and radish rhizospheres (Figs. 4.23, 4.25) and in the presence of tobacco callus (Tables 4.37, 4.39) than the parent strain. Tn5-insertion mutants of strain GR12-2R3 that did not utilize C sources such as arabinose, glycerol and glycine failed to promote canola root elongation (Bayliss et al. 1993). However, the plant growth promoting activity of the mutants is probably regulated by a variety

of different mechanisms and the nature of the mutagenized genes is still unknown (Glick 1995).

The detection of nucleotides in *n*-butanol fractions of 72-, 96- and 168-h samples of G20-18WT cultures indicated that they might be precursor metabolites that were quickly degraded as the lag period of cytokinin production ended. The monoclonal antibody J3-I-B3 of the ZR immunoassay cross-reacts 95.2% with the nucleotide ZRNT (Sigma Phytodetek-ZR insert) that could be present in 96 and 168 h-old sample extracts of G20-18WT. If that was the case, the data shown in Fig. 4.4 for those samples are estimates of both ZR and ZRNT, but the relative amounts of each metabolite were not quantified. However, as growth proceeded the amounts of ZR increased and ZRNT was not detected in 240 and 336 h-old samples. Thus, the estimates were not affected by the cross-reactivity between these two compounds.

As Z and DHZ are immediate precursors of ZR and DHZR, respectively, the lack of their detection in the TLC chromatograms is an indication that they were quickly metabolized (Fig 2.3). Hence, the cross-reactivities of Z and DHZ with the respective antibodies J3-I-B3 and J23-II-B1 of ZR and DHZR were not a concern in this case.

Although they were not quantified, Z7G and Z0G were detected on the TLC chromatograms of 14-d-old cultures of strain G20-18WT and its selected mutants. This indicated that the O-glucosyltransferase enzymes were active and competing for the same substrate with the Z-reductase and phosphorylase, which metabolize Z to produce DHZ and ZR, respectively.

The presence of an unknown metabolite at R_F =0.45 does not change the immunoassay estimates, because the metabolites having a high percent of cross-reactivity with IPA, ZR and DHZR were all included as standards in the TLC chromatograms and all have different R_F (Table 4.4). Bacteria have been shown to release cytokinin metabolites, which were not found in plants (MacDonald et al. 1986). It is possible that this unknown metabolite is such a cytokinin. This unidentified compound or group of cytokinins is another indication that strain G20-18WT can produce an array of cytokinin metabolites. These results are in contrast with those of Timmusk et al. (1999) who observed that a strain of *Paenibacillus polymyxa* isolated from the rhizosphere of wheat produced the cytokinin IPa at a concentration of 1.5 nM and an unknown cytokinin compound in late stationary phase (20 d). They used HPLC with on-line ultraviolet detection and a final step of GC-MS. The immunoassays performed in combination with TLC used in the present study helped to qualitatively characterize the type of cytokinins produced by the PGPR strains without requiring expensive equipment. Although, the electro-spray HPLC-MS technique did not provide accurate quantitative estimates of IPA, ZR and DHZR because internal radioactive standards were not used, these cytokinins were qualitatively detected and thus the results shown in Table 4.5 validated the immnunoassay estimates. These observations show that with the required standardization procedures immunoassays can give accurate estimates of cytokinins. Internal radioactive cytokinin standards require specially equipped

laboratory facilities, and could eventually be metabolized during the handling of the samples, reducing the accuracy of the estimates (Timmusk et al. 1999).

Muller et al. (1988) estimated higher amounts of IPA, ZR and DHZR in supernatants of a *P. fluorescens* strain isolated from maize rhizosphere after 3 d of growth than found in the present study. It is probable that cytokinin concentrations were higher because the medium contained 0.5 g L^{-1} of yeast extract which has been shown to contain large amounts of IPa and IPA and to be inappropriate for cytokinin studies (Jameson and Morris 1989). The data reported here are the first demonstrating that a *Pseudomonas* strain can produce several cytokinins during and after the stationary phase of growth.

The production of cytokinins by strain G20-18WT during the stationary phase of growth could be increased using the precursor Ade (Fig. 2.1) that can be found in root exudates (Bolton et al. 1993; Arshad and Frankenberger 1993). Although their activities were not determined, it can be speculated that the enzymes involved in cytokinin metabolism in this PGPR strain were substrate limited. At 96 h, production of IPA, which is an early metabolite, increased with the addition of the lowest Ade concentration (Fig. 4.9), but the production of DHZR was increased with the intermediate Ade concentration (Fig. 4.11). This is in agreement with the general metabolic pathway (Fig. 2.3), because, if the amount of Ade added is large enough, the initial increase in IPA will lead to an increase in other precursor metabolites and consequently in the amounts of DHZR produced by strain G20-18WT. As DHZR increased at 96 h (Fig 4.11) and ZR increased only after 168 h (Fig. 4.10) following addition of Ade, these

data are an indication that the metabolic pathway through DHZR is favored before that through ZR in strain G20-18WT. In contrast, *A. chrococcum* strain ATCC 9043 produced more ZR than DHZR when similar concentrations of Ade (10^{-5} M) in combination with isopentyl alcohol (10^{-3} M) were added to the culture medium (Nieto and Frankenberger 1989). This indicates that different PGPR strains have different metabolic pathways or that the same metabolic pathway is differently modified when cytokinin precursors are supplied.

The results presented here demonstrated that the precursor Ade could be used to increase the production of cytokinins by PGPR strain G20-18WT in the rhizosphere. Also, as Ade is a compound frequently found in the rhizosphere environment (Arshad and Frankenberger 1993), a complementary approach could be to screen plant cultivars based on the composition of their root exudates and select for Ade producers (Stenlid 1982).

5.2. *P. fluorescens* strain G20-18WT in association with Katepwa wheat.

The purpose of this study was to determine whether *P. fluorescens* strain G20-18WT could colonize and promote growth of Katepwa wheat. This work was based on laboratory, greenhouse and several years of field experiments from which this strain was selected as a promising PGPR for this cultivar of spring wheat (R. J. Rennie, personal communication). Although inconsistent responses were obtained in field experiments, they were similar to those observed for other PGPR (Okon and Labandera 1994; Boddey et al. 1986; Weller et al. 1988; Kloepper et al. 1989), particularly in wheat (Juhnke et al. 1987).

It has been demonstrated that the plant selects specific populations of fluorescent pseudomonads (Lemanceau et al. 1995; Miller et al. 1989). Bacterial populations of wheat rhizosphere have been reported to consist of up to 23% pseudomonads (Vagnerova et al. 1960; Sands and Rovira 1970; Miller et al. 1989). Therefore, other bacterial groups or species may be present in large numbers and may play a significant role in the rhizosphere (Miller et al. 1989).

A general concept is that beneficial PGPR are effective only when they successfully colonize and persist in the plant rhizosphere (Elliot and Lynch 1984). *P. fluorescens* strain G20-18WT can colonize the rhizosphere of Katepwa wheat at different stages of plant growth and in different growing conditions such as growth pouches in the laboratory (Table 4.9), small pots in the greenhouse (Table 4.11), or large pots in the growth chamber (Table 4.13). Although the seeds were surface-sterilized, the differences between the bacterial numbers counted on non-selective (PAF) and selective (PAF+Rif or PKS) media indicated that other bacterial populations were present in the seeds. Transmission through seeds is very common among phytopathogenic bacteria, which may enter seeds during the flowering process or via the stomata of the seed coat and via the vascular system (Goto 1992). The occurrence of diazotrophs in seeds has also been reported for *Azospirillum* spp. (Sundaran and Klucas 1988) and *Herbaspirillum* spp. (Baldani et al. 1992; Olivares et al. 1996) and *Pseudomonas* spp. (Pimentel et al. 1991; Gillis et al. 1991). Thus, these data showed that the RIF mutant of strain G20-18WT and the transconjugants were effective colonizers of the rhizosphere and they could grow inside the plant and

colonize the aerial tissues. This ability to colonize inner and more protected spaces of the plant was observed as a strategy used by epiphytic pseudomonads (Beattie and Lindow 1994), but no previous references have been found for non-pathogenic *P. fluorescens* strains. The numbers of bacteria counted on the root using selective media (Tables 4.11 and 4.13) were comparable to those obtained for effective rhizosphere colonizers of maize (Scher et al. 1984), for other *P. fluorescens* strains in wheat roots (Milus and Rothrock 1993; Miller et al. 1989) and for the association between Katepwa wheat and strain G20-18WT in a previous report (Volkmar and Bremer 1998).

P. fluorescens strain G20-18WT and its mutant RIF showed similar performance as both strains increased emergence of Katepwa wheat in small (Table 4.10) and large (Table 4.12) pots. Also, both strains had similar effects on root and shoot biomass in greenhouse conditions (Fig. 4.13), which were significantly higher than those obtained with the transconjugants that had a reduced ability to produce cytokinins in pure cultures (Figs. 4.1 and 4.2). As no differences in root and shoot colonization were observed, it is evident that other strain characteristics were involved in the type of inoculation responses described.

In this study, strain G20-18WT significantly increased the number of tillers and visible ears (Fig. 4.14), leaf area at both 70 and 90 d.a.p. and root biomass at 90 d.a.p. (Table 4.15) but no beneficial effects were observed on grain yield (Table 4.16). A previous study of this PGPR strain and Katepwa wheat showed no effects on root growth and on yield components (Volkmar and Bremer 1998).

It was suggested that the lack of consistency was related to changes in exudation pattern during the life cycle of the plant, leading to lower numbers of pseudomonads in the rhizosphere in the later phase of the plant growth cycle (Miller et al. 1989). However, there does not appear to be any evidence of an inhibitory effect by Katepwa wheat on strain G20-18WT or mutant RIF.

It was suggested that chemotaxis of *Pseudomonas* strains (Scher et al. 1985; Howie et al. 1987) and other PGPR (Soby and Bergman 1983; Barbour et al. 1991) towards seed exudates might be the first phase in the establishment of bacterial seed and root colonization. A broad range of compounds present in the rhizosphere can induce plant-*Pseudomonas* interactions. (Van Overbeek and Van Elsas 1994). However, although the composition of exudates from Katepwa was not determined in this work, the exudates did not show any effect on the growth of strain G20-18WT other than negative effects when high concentrations were used (Fig. 4.12).

A second phase of the colonization process is when bacteria spread locally, multiply and survive, avoiding displacement (Howie et al. 1987). Although significant effects were observed when certain PGPR isolated from other plants were used to inoculate wheat in different environmental conditions (Kapulnik *et al.* 1987; Barbieri et al. 1988; Bashan et al. 1989, 1990), the roots of a crop may be most effectively colonized by microorganisms, which were originally isolated from its rhizosphere (Heijnen et al. 1993; Nijhuis et al. 1993; Van Overbeek and Van Elsas 1994). Several studies have reported beneficial effects of PGPR isolated from the rhizosphere of wheat (Boddey et al.1986; Chanway et al. 1988;

De Freitas and Germida 1990; Garcia Salamone et al. 1990). Grayston et al. (1990) reported that certain PGPR strains isolated from rhizosphere of Katepwa wheat increased growth and grain-yield of this cultivar at two field sites in Saskatchewan but they did not promote growth of the related cultivar Neepawa. Although strain G20-18WT could colonize Katepwa wheat, it was isolated from an arctic grass and this could be the cause of the lack of yield responses.

The responses of Katepwa wheat to inoculation could be related to microbial cytokinin production in association with the plant. The observations of increased leaf area post-anthesis and numbers of visible ears at anthesis have been related to a longer period of chlorophyll retention (Nooden et al. 1990b; Van Staden et al. 1988) and higher cytokinin contents (Mok 1994; Hammerton et al. 1998). Although few studies have compared endogenous cytokinin quantities or sensitivity to exogenous cytokinin applications to cereals, cytokinin content of a variety of cereal tissues has been correlated with developmental or growth processes (Michael and Seiler-Kelbitsch 1972; Saha et al. 1986; Saavedra-Soto et al. 1988; Ambler et al. 1992; Morris et al. 1993). Some studies of cytokinin physiology have been concerned with cereal genetic variability. Takagi et al. (1989) identified different types of endogenous cytokinin metabolites during the life cycle of three rice varieties and showed that individual metabolites were present in roots and shoots, but the former always had higher levels and reached their maximum at different stages of development. He also observed that all the cytokinins in the ear reached a maximum level at the early growth stages, between heading and anthesis, suggesting that cytokinins may play determinant

roles in the development of the grain. Two small-kernel varieties of barley had a reduced cytokinin content compared to that measured in a variety which produced heavier grains (Michael and Seiler-Kelbitsch 1972). The cytokinin content of leaf exudates during grain ripening in two rice cultivars was significantly increased in the high-yielding cultivar and it was also correlated with extended chlorophyll retention in the leaves (Soejima et al. 1992). Five cultivars of wheat of contrasting stature and chlorophyll retention differed in cytokinin content which was correlated positively with chlorophyll retention, but it was not directly correlated to plant stature (Banowetz 1996).

No determinations of cytokinins in plant tissues of Katepwa wheat were done in this study because no significant increases in grain yield were obtained with G20-18WT inoculation.

The inconsistency of the grain yield results after inoculation with PGPR is a common observation and this is particularly true for wheat because this crop shows great homeostasis (Simpson 1990) that can dilute the inoculation effects during the stage between anthesis and physiological maturity of the grains. In this regard, Pierson and Weller (1994) reported that the use of certain combinations of fluorescent pseudomonads enhanced the growth and yield of wheat in fields infested with *Gaeumannomyces graminis* var. *tritici*, whereas other bacterial mixtures or strains used individually did not. A factor that contributes to an inconsistent performance is the variable production or inactivation *in situ* of the bacterial metabolites responsible for disease control (Weller et al. 1988; Weller and Thomashow 1993). *In vitro* production of

metabolites by *Pseudomonas* spp. depends on cultural conditions and *in situ* production is likely to be even more sensitive to the physical and chemical environment in the rhizosphere or inside the plant (Howie et al. 1987; Shanahan et al. 1992). This concept could be applied in studies of *Pseudomonas* PGPR as direct beneficial agents of plant growth and it could be the cause of the lack of response in grain yield of Katepwa wheat. Cytokinins produced initially during early stages of growth and development can be metabolized in different ways (Fig. 2.3) to those produced during later stages of the life of the plant. The level of active cytokinin at a particular site of action is influenced by the metabolism and there is also the possibility that physiological responses may be modulated by variations in the ability of cells to respond to a particular concentration of free cytokinin (Saunders 1994). Several authors have shown that a transient peak occurred in the endosperm mitotic index and in the concentrations of certain cytokinins (Z, ZR and IPA) within developing maize kernels at 9 days after pollination (Jones et al. 1990; Lur and Setter 1993; Carnes and Wright 1988). During this time the enzyme cytokinin oxidase increased in parallel with the mentioned cytokinins but only in central kernels that matured normally (Dietrich et al. 1995).

The formulated hypothesis could be further studied for Katepwa wheat if either a mixture of characterized cytokinin producer strains and/or one or more cytokinin over-producer mutants were used. However special consideration should be given to the origin of the strain when direct beneficial inoculation responses are the objective of the study.

5.3. PGPR strains in association with Raphanus sativus cv. Cherry Belle

Raphanus sativus (radish) has been identified as a plant system, which responds to exogenous applications of cytokinins and their metabolism has been partially studied by several authors (Letham 1971; Parker and Letham 1973; Radin and Loomis 1974; Wilson et al. 1974; Gordon et al. 1974; Entsch and Letham 1979). Kloepper and Schroth (1978) reported that specific *Pseudomonas* PGPR strains could colonize radish roots and increase plant growth by suppressing pathogens. Nieto and Frankenberger (1989) showed that the exogenous supply of cytokinin-precursors and inoculation with *Azotobacter chroococcum* promoted growth of radish cv. Cherry Belle. Radish cv. Cherry Belle was chosen as a whole plant system appropriate to study the effects of inoculation of *Pseudomonas* PGPR and cytokinin production.

Regardless of the experimental approach used, all the PGPR strains included in this work colonized the seeds and roots of radish (Figs. 4.16, 4.28, 4.30). These data showed that the first step to establish a beneficial and efficient relationship between plant and bacteria was accomplished. Thus, cytokinins produced by these PGPR could affect the growth of radish because their roots are heavily populated ($> 10^7$ cfu plant^{-1}) as reported for *Azospirillum* associated with graminaceous plants (Jagnow 1987; Fallik et al. 1989). Although all the strains were established on radish seeds and grew and colonized radish roots, they had different effects on plant growth. Strains GR12-2 and Ral-3 did not exert beneficial effects on radish growth under the experimental conditions tested. *Pseudomonas* strains G20-18WT, G8-32 and 63-28 increased emergence

and biomass of roots and shoots of radish seedlings (Tables 4.17, 4.23, 4.24). However, when the plants were grown to maturity, the best inoculation response was obtained with strain G20-18WT (Table 4.30). This is the first report of beneficial effects of *Pseudomonas* PGPR on growth of radish plants in the absence of pathogens.

Frankenberger and Arshad (1995) reported that only a few attempts have been made to evaluate the possibility of using cytokinins to improve plant growth and productivity. Most of the physiological effects attributed to cytokinins have been observed by exogenous applications of these PGRs. The studies using exogenous applications of cytokinins have one or more of the following limitations: Firstly, Z-type cytokinins differ in their metabolism from the more stable BAP and kinetin frequently used and hence their behaviour may not reflect that of the endogenous cytokinins (Letham 1994). Secondly, the concentrations applied have been higher than those encountered naturally and may even have been toxic when millimolar concentrations were applied (Trewavas 1987, 1992). Thirdly, the identities of the radioactive compounds in the shoot tissues cannot be completely established because it is difficult to quantify how much of an exogenously applied dose of a PGR is translocated in biologically active form to a target tissue (Palni et al. 1990).

This work is the first concerned with the study of exogenously applied cytokinins to a whole plant in a range of concentrations similar to those that occur endogenously and could be supplied by a PGPR associated with the plant.

The attributes of radish plants such as root and shoot length (Table 4.19) and emergence, biomass and RRSA (Tables 4.23, 4.24) were significantly increased by exogenous applications of Z and DHZR. Interestingly, inoculation of radish seeds with the selected PGPR strains showed similar responses to those obtained with exogenous applications of Z and DHZR concentrations lower than 50 nM. The effects of exogenous supply of Z (Table 4.23) were similar to those produced by *Pseudomonas* PGPR but DHZR effects on RRSA were significantly higher (Table 4.24). Riboside cytokinins appeared to predominate in xylem exudates and when cytokinin bases were supplied to roots, the corresponding ribosides (Fig. 2.3) were the only form in the xylem (Letham 1994). It is possible that Z is less efficient than DHZR because Z must be metabolized to DHZR to exert the same physiological effect.

Although xylem metabolites determined in stems following supply of labeled Z or ZR to the root tissues and translocation have been identified unambiguously in only a few plant systems, the most active cytokinins in plant tissues are Z, ZR, DHZ and DHZR (Letham 1994). However, it should be considered that metabolic differences exist not only among plant species but also at the organ and tissue levels (Jameson 1994).

Cytokinin metabolism of radish plants can be altered by inoculation with strain G20-18WT and resulted in significantly higher amounts of IPA, ZR and DHZR and different ratios between sterile and inoculated radish rhizospheres (Table 4.20). The average ratio of estimated cytokinins (IPA:ZR:DHZR) in pure cultures of strain G20-18WT was 79:7.5:12.5 while the average ratio in

inoculated rhizosphere of radish grown in GP was 51:10:39. These data (Table 4.20) show that amounts of IPA and DHZR change significantly as a result of the bacteria-plant interaction while the amounts of ZR were relatively constant. However, the patterns of production of ZR in pure cultures and in radish rhizosphere were different (Fig. 4.23).

Although roots and shoots showed different thresholds of response, the similarities in the responses to exogenous supply of IPA:ZR:DHZR combinations and inoculation with strain G20-18WT (Figs. 4.17, 4.18, 4.19) were clear indications that radish plants absorbed cytokinins and that this PGPR could supply types and concentrations of cytokinins similar to those exogenously provided to promote plant growth.

Only three cytokinin metabolites were quantitatively determined but they are strategically positioned in the metabolic pathway (Fig. 2.3) allowing one to obtain relevant information about the nature of this bacteria-plant interaction and the growth promotion by cytokinins produced by strain G20-18WT. As IPA is the second metabolite in the metabolic pathway (Fig. 2.3), and all the other cytokinin metabolites can be synthesized from it, the increased production of IPA could directly promote plant growth. However, DHZR is another active metabolite, which was significantly increased in radish rhizosphere and possibly absorbed by the plant (Fig. 4.24).

Data obtained from GP (Figs. 4.20, 4.21, 4.22) and small-pot (Tables 4.27, 4.28) experiments to compare strain G20-18WT and its mutants showed that the transconjugants did not increase radish growth. In addition, cytokinin analyses

of radish rhizospheres (Figs. 4.23, 4.24, 4.25) and plant tissues (Table 4.29) showed that these mutants had reduced ability to produce cytokinins in both pure cultures and radish rhizosphere. Amounts of ZR and DHZR in roots and shoots of radish plants inoculated with strains G20-18WT and RIF were similar but IPA amounts were significantly higher in G20-18WT-inoculated plants (Table 4.29).

Roots and shoots of radish plants responded differently to exogenous Z (Table 4.25). Plants inoculated with strain G20-18WT or exogenously supplied with 0.5 nM Z had different amounts of IPA and DHZR in their tissues, (Table 4.26), indicating that they metabolized these two cytokinins differently. As Z is not a precursor of IPA synthesis, it was expected that the amounts of IPA in roots of radish supplied with 0.5 nM Z (Table 4.26) would be similar to those in roots of control plants. However, the increased amounts of IPA in shoots of plants supplied with Z could only be explained if the exogenous supply of Z activates the *de novo* synthesis of IPA. Similarly, exogenous cytokinins elevated the endogenous cytokinin levels in several cytokinin-requiring tissues (Hansen et al. 1985, 1987) and in excised watermelon cotyledons (Marziani et al. 1991) suggesting an autoinductive mechanism that induces synthesis of cytokinins (Letham 1994). Although no differences were observed between the plant responses to exogenously supplied Z (0.5 nM) and inoculation with strain G20-18WT (Table 4.25) significant differences in the metabolism of DHZR were observed and the inoculated plants contained significantly higher amounts of

this cytokinin. The significance of DHZR in root-to-shoot translocation has been suggested elsewhere (Letham 1994).

DHZ and DHZR appear to be the major cytokinins in tissues with a high cytokinin oxidase activity (Nandi et al. 1990). Initial metabolites of xylem cytokinin formed in the leaves and stem of de-rooted radish include nucleotides, which are subsequently converted to glucosides and base-type compounds (Fig. 2.3) (Parker and Letham 1973) and are not accepted by cytokinin oxidases (Laloue and Fox 1989; Van Staden and Mooney 1988). In many tissues, accumulations of relatively high levels of Z and ZR, which are very susceptible to cytokinin oxidase, are indicative of some form of compartmentalization (Summons et al. 1979). The high amounts of ZR and DHZR in radish plants supplied with both exogenous and bacterial cytokinins (Table 4.26) are indications that compartmentalization and synthesis of oxidase-resistant metabolites are both involved in the cytokinin metabolism of radish cv. Cherry Belle. The Z7G and DHZ7G, which occur as endogenous cytokinins were detected following the application of ^{3}H-Z and ^{3}H-DHZ to derooted radish seedlings where N-glucosylation was the predominant form of metabolism (Parker and Letham 1973; McGaw et al. 1984). As the amounts of ZR in radish plants supplied with both exogenous and bacterial cytokinins were similar, it is possible that cytokinins supplied by PGPR associated with roots could be taken up and metabolized in the same way (Fig. 2.3). Cytokinin-7-glucosyltransferases have been extracted from radish cotyledons (Jameson 1994).

Clearly, cytokinins supplied by strains G20-18WT and RIF maintained significantly more active chlorophyll units in cotyledons of radish seedlings than those in control and transconjugant-inoculated plants (Table 4.28). This feature has been related to delay of senescence elsewhere (Nooden et al. 1990a; Nooden and Letham 1993). Participation of cytokinins in the control of leaf senescence is a firmly established concept in plant physiology (Letham 1994). McGaw et al. (1985) have shown that the O-glucoside moiety can be cleaved when O-glucosides of DHZ, Z and DHZR were fed to de-rooted radish seedlings. Similarly, Letham and Gollnow (1985) detected substantial amounts of Z7G and DHZ7G in radish cotyledons, but O-glucosides of these compounds were not detected, indicating the conversion of stored O-glucosides to their corresponding bases prior to N-glucosylation. Although in this work, O-glucosides were not determined in radish tissues, it is possible that the increased amounts of ZR and DHZR in plants inoculated with strain G20-18WT were associated with the production of their O-glucosides as storage forms. This situation would not change the estimates of IPA, ZR and DHZR obtained in this work because the cross-reactivity between them and their respective O-glucosides are indicated as null or less than 0.08% (Sigma Phytodetek Inserts).

Although DHZ was not detected in supernatants of strain G20-18WT, it is possible that the increased amounts of DHZR in plant tissues were the result of the metabolism of ZR exogenously supplied by this strain in the rhizosphere of radish via DHZ (Fig. 2.3). Taylor et al. (1990) found that DHZ was the main

labeled cytokinin in phloem sap exuded by pods of lupin plants that had been supplied with [^{3}H]-ZR through the roots.

These data are the first to show a direct correlation between plant growth and production of cytokinins by a *Pseudomonas* PGPR in contact with a plant in a non-symbiotic and non-parasitic association. This suggests that radish cv. Cherry Belle could be used as a whole-plant bioassay for screening of cytokinin-producing rhizobacteria.

5.4. *Pseudomonas* PGPR in Association with Tobacco Callus

In this work, the tobacco callus bioassay (TCB) was standardized to test the ability of PGPR to produce cytokinins in the presence of plant tissues. Only specific combinations of stock callus and culture media were appropriate for this purpose. Usually TCB has been conducted to detect cytokinin concentrations higher than 500 pmol L^{-1}. In this study, using the combination of stock callus and culture media [SD+0]/[0+0], Z concentrations as low as 5 pmol L^{-1} could be detected (Fig. 4.33). This feature made the TCB a highly sensitive approach to study the production of cytokinins by PGPR in the presence of plant tissues.

TCB has been used to study the metabolism of exogenously supplied cytokinin compounds (Mok 1994; Letham 1994). A new feature of the TCB reported in this work is that the tobacco callus tissues were standardized to respond to cytokinin excreted by active living cultures of PGPR. As the culture media did not contain any cytokinin, one can assume that PGPR and/or tobacco tissues produced any cytokinins present in the TCB plates.

Cytokinin precursors, IPP and AMP, (Fig. 2.3), from other metabolic pathways (Kumar and Lonsane 1989; Murai 1994), could be provided by the bacteria or tobacco tissues. Two biosynthetic pathways have been reported for cytokinins: the *de novo* pathway, (Fig. 2.3) and the tRNA pathway (Chen and Ertl 1994) and both have been detected in cytokinin-requiring tobacco tissues (Murai 1994). Both pathways probably use similar enzymes in their initial steps. Although there is no report of the total purification of the isopentenyltransferase enzyme (Fig 2.3) in plants, it has been partially purified from several plant tissues, including tobacco cell cultures (Chen and Ertl 1994). A similar enzyme catalyzing the biosynthesis of IPA in tRNA was called tRNA N^6-isopentenyltransferase and its activity was reported in crude cell free extracts from yeast, *Lactobacillus*, corn, tobacco callus and animal tissues (Taller 1994). However, free cytokinins are limited to higher plants and microorganisms that form association with higher plants (Murai 1994; Taller 1994).

Regardless of the combination of stock callus and culture media used, all *Pseudomonas* PGPR tested in this study increased fresh weight and produced cytokinins in the presence of tobacco callus (Tables 4.33 and 4.34). However, significant differences among strains were detected only when ZE-cfu were analyzed. Nieto and Frankenberger (1989) indicated that the amount of ZE produced by a PGPR had little meaning unless numbers, optical density or dry weights of bacteria were known.

The inoculation technique used in the TCB experiments reported here, involving no contact between PGPR cells and tobacco callus tissues, was

successful because the tobacco callus tissues could effectively use cytokinins excreted by PGPR strains to grow. When stock callus was directly inoculated so that the PGPR cells and tobacco callus were in contact, death of the tissue occurred in a few days (data not shown).

As the metabolism of cytokinins in cultures of tobacco cells in liquid medium is characterized by a very active absorption during the first period of growth, intracellular concentrations of the various cytokinin derivatives are usually modified by metabolic interconversions and by dilution directly linked to cell division (Gawer et al. 1977). In contrast, regardless of the inoculation treatments, tobacco callus in this work, grew slowly during the first 7 d and after that, exponentially up to the end of the TCB (Figs. 4.39 and 4.40). Except for ZR production in T and T+G20-18WT plates, the amounts of cytokinins excreted into the medium increased during the assay (Tables 4.37, 4.38 and 4.39).

In this study, it has been demonstrated that strain G20-18WT can excrete cytokinins and increase growth of tobacco callus tissues in culture media with and without addition of glucose (Fig. 4.38). Numbers of PGPR counted in T+B plates at the end of the assay indicated that bacterial cells could grow in these culture conditions (Tables 4.32 and 4.35). Interestingly, although bacterial numbers were significantly higher (Table 4.32), the addition of PAF ingredients reduced the sensitivity of tobacco callus to cytokinins (Figs. 4.37 and 4.38).

Both direct determinations of [IPA+ZR+DHZR] by immunoassays (Fig. 4.2) and ZE-cfu estimates obtained using TCB (Fig. 4.35) demonstrated that strain

G20-18WT could produce significantly higher amounts of cytokinins than the selected mutants. However, amounts estimated by immunoassays were higher than those obtained from the standard dose-response equation of the TCB. The same situation was observed when amounts of cytokinins were estimated in cultures supernatants of G20-18WT with and without addition of Ade (Figs. 4.8 and 4.35). This could be related to the possibility that the stock callus had an optimum for cytokinins and extra cytokinins did not enhance the growth of tobacco callus. Another alternative explanation could be related to the type of cytokinins present in the supernatants. Letham (1978) reported that large and stable N^6-substituents abolish cytokinin activity. Hence, ZOG and related O-glucosides are unlikely to be active per se; the glucosyl moiety of exogenous O-glucosides is known to be cleaved in plant tissues (Letham and Palni 1983) and the activity of these metabolites is a consequence of this cleavage (Kaminek 1992; Jameson 1994; Frankenberger and Arshad 1995). Certain cytokinins such as 7- and 9-glucosyl derivatives have been reported to show a reduced activity in bioassays (Letham et al. 1983). Also, it has been reported that cytokinin ribosides are less active in TCB and radish cotyledon bioassays than the corresponding bases (Letham 1978; Matsubara 1980). However, these authors did not include DHZ or DHZR in the experiments described. Interestingly, these cytokinins have been shown to have a significant role in the production of cytokinins by strain G20-18WT (Table 4.39) and their presence in the TCB plates is probably one of main causes of the significant increases in fresh weight observed in T+G20-18WT plates (Fig. 4.39). Z7G and ZOG were probably

present in supernatants of pure bacterial cultures as they were detected in TLC chromatograms.

The total amounts of cytokinins detected by TCB in supernatants of pure cultures of strain G20-18WT (Fig. 4.35) were higher than those detected in T+B plates of this strain estimated using the equation of the standard dose-response equation (Fig. 4.39). These apparent discrepancies could be an indication that the tobacco callus was selectively metabolizing and using the cytokinins excreted by the bacteria into the medium for growth.

The combination [SD+0]/[SD+0] of culture media was less sensitive to Z concentrations lower than 500 pmol L^{-1} than the combination [SD+0]/[0+0]. As PGPR produced less than 500 pmol L^{-1} of ZE and the culture medium contained 2 mg L^{-1} of auxins supplied as 2,4-D (Murashige and Skoog 1962), one possible explanation is that the amounts of auxins in callus grown using the combination [SD+0]/[SD+0] were too high to respond when such low cytokinin concentrations were provided. In other words, the callus needed higher amounts of cytokinins than those supplied by the PGPR to increase their fresh weight. The higher sensitivity to the low amounts of cytokinins supplied by the PGPR strains observed for tobacco callus grown using the combination [SD+0]/[0+0] could be related to the need for sufficient auxins in the tissues to respond to cytokinins and to increase DNA replication and mitosis (Jacqmard et al. 1994). In pea root cortical explants, tobacco cell suspensions and protoplasts, it was found that the presence of auxins in the culture medium for some time before the

cytokinins were supplied was a prerequisite for the latter to affect cell-cycle processes (Meyer and Cooke 1979).

Maintenance of the correct ratio of auxin to cytokinin for the specific growth requirement in a given plant system requires fine control of the cytokinin concentration (Klee and Estelle 1991). The presence in tissues of relatively large amounts of different cytokinins with widely differing activities has been suggested as part of a sophisticated mechanism for controlling the active cytokinin concentration requirements (Shaw 1994). Similar comments could apply to IAA, which also occurs as various conjugates that may be hydrolyzed to release IAA during growth processes (Cohen and Bandurski 1982). Cytokinin degradation by oxidase enzymes is also an important component process in the metabolic network that controls the levels of cytokinin metabolites and their distribution in plant systems (Armstrong 1994). Several studies suggested that an increase in auxin concentrations in the culture medium of tobacco pith explants doubled the degradation of ZR (Palni et al. 1988). Hansen et al. (1988) observed that ZR accumulation in cytokinin-autotrophic tobacco cells was blocked by the addition of auxin to the culture medium. In contrast, although the endogenous cytokinin content of soybean tissues was inversely correlated with the auxin content of these tissues, the cytokinin oxidase activity was not significantly changed (Wyndaele et al. 1988). Motyka and Kaminek (1992) found that transient increases in the auxin concentrations supplied to tobacco callus cultures failed to elicit any increase in the cytokinin oxidase of callus. Although IAA was excreted into the culture medium during the TCB, no

differences were observed among PGPR strains and T+B plates contained the sum of the amounts estimated in T and B plates (Table 4.40). For that reason, the differences observed in the metabolism of ZR between B and T+B plates (Table 4.38) could not be attributed to changes in IAA metabolism.

Although it is probable that small amounts of gibberellins were excreted into the medium (Fig. 4.40), it is unlikely that they could have played any role in the promotion of growth observed in the TCB experiments as no differences were observed among treatments.

Although exogenous cytokinins induce cytokinin oxidase activity in cultured tissues, which could result in a degradation of endogenous cytokinins (Motyka and Kaminek 1990), it is probable that this did not happen in T+B plates with the mutants, which contained small amounts of cytokinins (Tables 4.37, 4.38 and 4.39). However, this could have happened in T+G20-18WT plates that contained large amounts of IPA and DHZR. Cytokinin degradation in plant tissues appears to be a developmentally regulated process and cytokinin oxidases have a significant role in specific processes and events of growth and development (Armstrong 1994). Accordingly, the T+G20-18WT plates showed the highest FWI values and cytokinin concentrations.

Reduced amounts of cytokinins such as those produced by the transconjugants showed a significant effect on FWI (Fig. 4.39). However, tobacco callus grown in the presence of strain G20-18WT or mutant RIF in the plates had similar and significantly higher FWI than calluses grown on plates with the transconjugants (Fig. 4.39). The amounts of ZE-cfu estimated from the

standard line equation also reflected the different performance of PGPR in the TCB (Table 4.36).

Interestingly, cytokinin production by PGPR strains was significantly different in B and T+B plates (Tables 4.37, 4.38 and 4.39). As in pure cultures in liquid MM+Gl medium, IPA was the major cytokinin detected in pure cultures on plates containing agar medium [0+0], followed by DHZR and ZR in that order. The lower amount of IPA and DHZR produced by the transconjugants in B plates are indications that they have altered activities of several enzymes in the metabolic pathway (Fig. 2.3). The differences in the amounts of cytokinin estimated in liquid MM+Gl and agar [0+0] media could be attributed to the effects that the culture conditions had on cytokinin production. The lack of auxin availability in medium [0+0] and the reduced auxin concentration in the tobacco tissues was probably an important factor regulating the production of cytokinins by PGPR strains in TCB plates.

In most cases the amounts of cytokinins in T plates were different from those in T+B plates. Except for concentrations of DHZR in T+G20-18WT plates, IPA, ZR and DHZR concentrations in T+B plates at 7 d were several orders of magnitude lower than those estimated in T plates. This is probably an indication that cytokinins produced by PGPR during the first 7 d of the TCB were quickly used by the tobacco callus to grow. This rapid uptake of cytokinins produced by PGPR probably occurred throughout the assay, but significant interactions between cytokinin and strains were observed. The large amounts of DHZR in T+G20-18WT plates are evidence of the significant role of this

cytokinin in the growth of tobacco callus. However the interaction with other cytokinins and the time they were produced may play a role in the promotion of tobacco callus growth because no significant effects were observed when this cytokinin was supplied alone to the culture medium in standardization experiments (data not shown).

In these circumstances, the amounts of cytokinins present in TCB plates represent the balance of cytokinins resulting from the production, assimilation and excretion of metabolites by both PGPR and tobacco callus tissues. As mentioned, the three cytokinins estimated by immunoassays are strategically located in the general biosynthetic pathway (Fig. 2.3) and knowledge of their concentrations in TCB plates gave fundamental information to increase the understanding of the beneficial effects exerted by certain PGPR on plant tissues.

Clearly in this study, it was demonstrated that PGPR associated with plant tissues can produce cytokinins and the result of the interaction between bacteria and plant tissues is a system with altered cytokinin metabolism. This system could be used to study plant growth promotion by PGPR and/or to study cytokinin metabolism using levels of cytokinins that are similar to those present in plant tissues.

5.5. Conclusions

In this work two plant systems, radish plants and tobacco callus, were shown to be sensitive to exogenous supply of cytokinins and responded to inoculation and cytokinins supplied by *Pseudomonas* PGPR. Wheat inoculation responses

involved significant increases in emergence, root and shoot biomass, number of tillers, visible ears and leaf area post-anthesis. These effects can also be induced by exogenous cytokinins. However wheat plants may need to be inoculated with PGPR strains that were isolated from the rhizosphere of the cultivar to be investigated in order to obtain grain-yield responses.

The formulated hypothesis can be accepted on the basis of the comparison between the inoculation effects produced by *P. fluorescens* strain G20-18WT and the Tn5-insertion mutants. Because of the intimate contact that strain G20-18WT established with the roots of wheat and radish, and the increase in tobacco callus growth observed in the presence of three selected PGPR strains, the data obtained in this work demonstrated that microbial cytokinins produced in the presence of plant tissues could directly promote plant growth.

The amounts of IPA, ZR and DHZR produced by *Pseudomonas* PGPR strains in pure cultures and in the presence of plant tissues were different. The rhizosphere of radish plants or the presence of tobacco callus modified the type and amounts of cytokinins produced by the studied strains. Although an array of other cytokinin metabolites was probably produced, the strategic location in the biosynthetic pathway of the three cytokinins (Fig 2.3) measured allowed for interpretation and analysis of cytokinin metabolism by the selected *Pseudomonas* PGPR strains in pure cultures and in association with plant tissues.

Although IAA was detected in bacterial supernatants (Table 4.6) and TCB plates (Table 4.40) no significant differences were observed among PGPR

strains. Similarly, supernatants obtained from GP with radish plants inoculated with G20-18WT or pure cultures of this strain (Fig. 4.26) did not show differences in the amounts of IAA present in the GP after 4 and 7 d. TCB plates had higher levels of IAA and cytokinins than both pure cultures and radish GP. Thus, these data suggest that the positive effects of strain G20-18WT on plant growth were not associated with the production of IAA or gibberellin-like substances because this strain and the mutants produced similar amounts in both pure cultures and in the presence of plant tissues.

In summary, this research showed that *P. fluorescens* strain G20-18WT could directly increase radish and tobacco callus growth because of its ability to produce cytokinins. Cytokinin metabolism of radish plants was altered by inoculation with strain G20-18WT resulting in significantly higher amounts of cytokinins in root and shoot tissues and different cytokinin ratios between sterile and inoculated radish rhizospheres. The standardized TCB was able to detect cytokinin concentrations as low as 5 pmol L^{-1} and may be appropriate for initial screening of cytokinin-producing PGPR in the presence of plant tissues. Radish cv. Cherry Belle grown in GP with MM+Gl medium was a sensitive whole-plant bioassay for screening of cytokinin-producing PGPR and also testing their ability to colonize the rhizosphere.

Cytokinin production by PGPR may have potential as an innovative alternative to enhance plant growth and as a sustainable approach to improve the yield and quality of agricultural crops. To obtain that goal, cytokinin-producing strains should be isolated from associated crops to ensure that strains adapted to

the specific crop and environment are selected. Following this, strains should be screened in greenhouse experiments and in field trials under different environmental conditions. This approach should provide the information required to develop cytokinin-producing PGPR inoculants for practical application by the farmer.

REFERENCES

Allen, M.F., Moore, T.S., and Christensen, M. 1980. Phytohormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae: I. Cytokinin increases in host plant. Can. J. Bot. **58**: 371-374.

Aloni, R. 1987. Differentiation of vascular tissues. Ann. Rev. Plant Physiol. **38**: 179-204.

Ambler, J.R., Morgan, P.W., and Jordan, W.R. 1992. Amounts of zeatin and zeatin riboside in xylem sap of senescent and nonsenescent sorghum. Crop Sci. **32**: 411-419.

Apelbaum, A., and Burg, S.P. 1972. Effect of ethylene on cell division and deoxyribonucleic acid synthesis in *Pisum sativum*. Plant Physiol. **50**: 117-123.

Armstrong, D.J. 1994. Cytokinin oxidase and regulation of cytokinin degradation. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 139-154.

Arshad, M., and Frankenberger, W.T., Jr. 1990. Microbial production of plant hormones. Plant Soil **133**: 1-8.

Arshad, M., and Frankenberger, W.T., Jr. 1993. Microbial production of plant growth regulators. *In* Soil microbial ecology. Applications in agricultural and environmental management. *Edited by* F.B. Metting, Jr. Marcel Dekker, Inc., New York. pp. 307-343.

Azcon, R., and Barea, J.M. 1975. Synthesis of auxin, gibberellins and cytokinins by *Azotobacter vinelandi* and *Azotobacter beijerinckii* related to effects produced on tomato plants. Plant Soil **43**: 609-619.

Azcon-Aguilar, C., and Bago, B. 1994. Physiological characterisitics of the host plant promoting an undisturbed functioning of the mycorrizal symbiosis. *In* Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. *Edited by* S. Gianinazzi and H. Schuepp. ALS, Birkhauser Verlag. Basel, Switzerland. pp. 47-60.

Badenoch-Jones, J., Summons, R.E., Djordjervic, M.A., Shine, J., Letham, D.S., and Rolfe, B.G. 1982. Mass spectrometric quantification of indole-3-acetic acid in *Rhizobium* culture supernatants; relation to root curling and nodule initiation. Appl. Environ. Microbiol. **44**: 275-280.

Balandreu, J., and Knowles, R. 1978. The rhizosphere. *In* Interactions between nonpathogenic soil microorganisms and plants. *Edited by* Y.R. Dommergues and S.V. Krupa. Elsevier Scientific Publishing, New York. pp. 243-268.

Baldani, V.L.D., Baldani, J.I., Olivares, F., and Döbereiner, J. 1992. Identification and ecology of *Herbaspirillum seropedicae* and the closely related *Pseudomonas rubrisubalbicans*. Symbiosis **13**: 65-73.

Banerjee, S.N. 1968. DNA synthesis in the root meristem cells of *Zea mays* dwarf mutants. Plant Cell Physiol. **9**: 577-583.

Banowetz, G.M. 1994. Immunoanalysis of cytokinins. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 305-316.

Banowetz, G.M. 1996. Cultivars of hexaploid wheat of contrasting stature and chlorophyll retention differ in cytokinin content and responsiveness. Ann. Bot. **79**: 185-190.

Barbieri, P., Bernardi, A., Galli, E., and Zanetti, G. 1988. Effects of inoculation with different strains of *Azospirillum brasilense* on wheat root development. *In* *Azospirillum* IV. Genetics, physiology, ecology. *Edited by* W. Klingmuller. Springer-Verlag, Berlin. pp. 181-188.

Barbour, W.M., Hattermann, D.R., and Stacey, G. 1991. Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. Appl. Environ. Microbiol. **57**: 2635-2639.

Barea, J.M. 1997. Mycorrhiza/bacteria interactions in plant growth promotion. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 150-158.

Barea, J.M., and Brown, M.E. 1974. Effects on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. **37**: 583-593.

Barea, J.M., Navarro, E., and Montoya, E. 1976. Production of plant growth regulators by rhizosphere-solubilizing bacteria. J. Appl. Bacteriol. **40**: 129-134.

Bashan, Y., and Levanony, H. 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbiol, **36**: 591-608.

Bashan, Y., and Holguin, G. 1997a. *Azospirillum*/plant relationships: environmental and physiological advances 1990-1996. Can. J. Microbiol. **43**:

103-121.

Bashan, Y., and Holguin, G. 1997b. Short- and medium-term avenues for *Azospirillum* inoculation. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 130-149.

Bashan, Y., Ream, Y., Levanony, H., and Sade, A. 1989. Non-specific responses in plant growth, yield, and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. Can. J. Bot. **67**:1317-1324.

Bayliss, C., Lasby, C., Wood, J., Lifshitz, R., and Brown, G. 1993. Mutant derivatives of *Pseudomonas putida* GR12-2R3 defective in nutrient utilization or cell surface structures show reduced ability to promote canola root elongation. Can. J. Microbiol. **39**: 1111-1119.

Beattie, G.A., and Lindow, S.E. 1994. Survival, growth, and localization of epiphytic fitness mutants of *Pseudomonas syringae* on leaves. Appl. Environ. Microbiol. **60**: 3790-3798.

Belding, R.D., and Young, E. 1989. Shoot and root temperature effects on xylary cytokinin levels during budbreak in young apple trees. Hortsci. **24**: 115-121.

Benhamou, N., Kloepper, J.M., Quadt-Hallman, A., and Tuzun, S. 1996. Induction of defense-related ultrastructural modification in pea root tissues inoculated with endophytic bacteria. Plant Physiol. **112**: 919-929.

Bergounioux, C., Perennes, C., Brown, S.C., Sarda, C., and Gadal, P. 1988. Relation between protoplast division, cell-cycle stage and nuclear chromatin structure. Protoplasma **142**: 127-138.

Biddington, N.L., and Thomas, T.H. 1973. A modified *Amarathus*-betacyanin bioassay for the rapid determination of cytokinin in plant extracts. Planta **111**: 183-186.

Blackman, P.G., and Davies, W.J. 1985. Root to shoot communication in maize plants of the effects of soil drying. J. Exp. Bot. **36**: 39-48.

Boddey, R.M., Baldani, V.L. D., Baldani, J.I., and Dobereiner, J. 1986. Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field grown wheat. Plant Soil **90**: 265-292.

Boerjan, W., Genetello, C., Van Montagu, M., and Inze, D. 1992. A new bioassay for auxins and cytokinins. Plant Physiol. **99**: 1090-1098.

Bolton, H., Fredrickson, J.K., and Elliot, L.F. 1993. Microbial ecology of the rhizosphere. Microbial production of plant growth regulators. *In* Soil microbial ecology. Applications in agricultural and enviromental management. *Edited by*, F.B. Metting, Jr. Marcel Dekker, Inc., New York. pp. 27-63.

Brinegar, C. 1994. Cytokinin binding proteins and receptors. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 217-232.

Brock, T.G., and Cleland, R.E. 1990. Biophysical basis of growth promotion in primary leaves of *Phaseolus vulgaris* L. by hormones versus light. Planta **182**: 427-431.

Brock, T.D., Madigan, M.T., Martinko, J.M., and Parker, J. 1994. Biology of Microorganisms, Seventh Edition. Prentice-Hall, Inc., Upper Saddle River, NJ.

Bromfield, E.S., Lewis, D.M., and Barran, L.R. 1985. Cryptic plasmid and rifampicin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. J. Bacteriol. **164**: 410-413.

Brown, M.E. 1974. Seed and root bacterization. Ann. Rev. Phytopathol. **12**: 181-197.

Brzobohaty, B., Moore, I., and Palme, K. 1994. Cytokinin metabolism: implications for regulation of plant growth and development. Plant Mol. Biol. **26**: 1483-1497.

Burch, L., and Stuchbury, T. 1987. Activity and distribution of enzymes that interconvert purine bases, ribosides and ribotides in tomato plants and possible implications for cytokinin metabolism. Physiol. Plant. **69**: 283-289.

Carley, H., and Watson, R. 1966. A new gravimetric method for estimating root-surface area. Soil Sci. **102**: 289-291.

Carmi, A., and Van Staden, J. 1983. Role of roots in regulating the growth rate and cytokinin content of leaves. Plant Physiol. **73**: 76-79.

Carnes, M.G., and Wright, M.S. 1988. Endogenous hormone levels of immature corn kernels of A188, Missouri-17 and Dekalb XL-12. Plant Sci. **57**: 195-203.

Challice, J.S. 1975. Separation of cytokinins by high-pressure liquid chromatography. Planta **122**: 203-207.

Chanway, C.P., Holl, F.B., and Nelson, L.M. 1988. Cultivar specific growth promotion of spring wheat (*Triticum aestivum* L.) by co-existent *Bacillus* species. Can. J. Microbiol. **34**: 925-929.

Chanway, C.P., Hynes, R., and Nelson, L.M. 1989. Plant growth-promoting rhizobacteria: effects on growth and nitrogen fixation of lentil (*Lens esculenta* Moench) and pea (*Pisum sativum* L.). Soil Biol. Biochem. **21**: 511-517.

Chen, C.M. 1982. Cytokinin biosynthesis in cell-free systems. *In* Plant growth substances. *Edited by* P.F Wareing. Academic Press, London. pp. 155-164.

Chen, C.M., and Ertl, J.R. 1994. Cytokinin biosynthetic enzymes in plants and slime mold. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 81-85.

Cohen, J.D., and Bandursky, R.S. 1982. Chemistry and physiology of bound auxins. Ann. Rev. Plant Physiol. **33**: 403-408.

Compeau, G., Al-Achi, B.J., Platsouka, E., and Levy, S. 1988. Survival of ripampicin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. **54**: 2432-2438.

Constantinidou, H.A., Steele, J.A., Kozlowski, T.T., and Upper, C.D. 1978. Binding specificity and possible analytical applications of the cytokinin-binding antibody, anti-N^6-benzyladenosine. Plant Physiol. **62**: 968-974.

Crowell, D.N., and Amasino, R.M 1991. Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. Plant Physiol. **95**: 711-715.

Crowell, D.N., and Amasino, R.M. 1994. Cytokinin and plant gene regulation. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 233-242.

Curl, E.A., and Truelove, B. 1986. The Rhizosphere. Springer-Verlag, New York.

Das, N.K., Patau, K., and Skoog, F.M. 1956. Initiation of mitosis and cell division by kinetin and indole acetic acid in excised tobacco pith tissue. Physiol. Plant. **9**: 640-652.

Dauphin-Guerin, B., Teller, G., and Durand, B. 1980. Different endogenous cytokinins between male and female *Mercurialis annua*. Planta **148**: 124-130.

De Freitas, J.R., and Germida, J.J. 1990. Plant growth promoting rhizobacteria for winter wheat. Can. J. Microbiol. **36**: 265-272.

De Freitas, J.R., Germida, J.J., and Hnatowich, G.L. 1997. Growth and yield response of canola to bacterial inoculants: a three year field assessment. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by*

A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino, Nakanishi Printing, Sapporo, Japan. pp. 209-211.

De Leij, F.A.A.M., and Lynch, J.M. 1997. Functional diversity of the rhizosphere. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino, Nakanishi Printing, Sapporo, Japan. pp. 38-43.

Dietrich, J.T., Kaminek, M., Blevius, D., Reinbott, T.M., and Morris, R.O. 1995. Changes in cytokinins and cytokinin oxidase activity in developing maize kernels and the effects of exogenous cytokinin on kernel development. Plant Physiol. Biochem. 33: 327-336.

Döbereiner, J., and Pedrosa, F.O. 1987. Nitrogen-fixing bacteria in non-leguminous crop plants. Brock-Springer Series in Contemporary Biology. Madison, Wis., USA.

Dommergues, Y.R. 1978. The plant-microorganism system. *In* Interactions between nonpathogenic soil microorganisms and plants. *Edited by* Y.R. Dommergues and S.V. Krupa. Elsevier Scientific Publishing, New York. pp. 1-37.

Dosselaere, F., Vande Broek, A., Lambrecht, P., De Troch, E., Prinsen, E., Okon, Y., Keijers, V., and Vanderleyden, J. 1997. Indole-3-acetic acid biosynthesis in *Azospirillum brasilense*. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino, Nakanishi Printing, Sapporo, Japan. pp. 306-309.

Doumas, P., Bonnet-Masimbert, M., and Zaerr, J.A. 1989. Evidence of cytokinin bases, ribosides and glucosides in roots of Douglas-fir, *Pseudotsuga menziesii*. Tree Physiol. 5: 63-69.

Dowling, D. N., and O'Gara, F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends Biotech. 12: 133-141.

Durand R. and Durand, B. 1994. Cytokinins and reproductive organogenesis in *Mercurialis*. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 295-304.

Elliot, L.F., and Lynch, J.M. 1984. Pseudomonads as a factor in the growth of winter wheat (*Triticum aestivum* L.). Soil Biol. Biochem. 16: 2793-2799.

Entsch, B., and Letham, D.S. 1979. Enzymatic glucosylation of the cytokinin, 6-benzylaminopurine. Plant Sci. Lett. 14: 205-212.

Evans, P.T., and Malmberg, R.L. 1989. Do polyamines have roles in plant development? Ann. Rev. Plant Physiol. Plant Mol. Biol. **40**: 235-241.

Fallik, E., Okon, Y., Epstein, E., Goedman, A., and Fischer, M. 1989. Identification and quantification of IAA and IBA in *Azospirillum brasilense* inoculated maize roots. Soil Biol. Biochem. **21**: 147-153.

Feldman, L.J. 1979. Cytokinin biosynthesis in roots of corn. Planta **145**: 315-421.

Fisher, R.F., and Long, S.R. 1992. *Rhizobium*-plant signal exchange. Nature (London) **357**: 655-660.

Fletcher, R. A., Kallidumbil, V., and Steele, P. 1982. An improved bioassay for cytokinins using cucumber cotyledons. Plant Physiol. **69**: 675-681.

Fosket, D.E., Volk, M.J., and Goldsmith, J. 1977. Polyribosome formation in relation to cytokinin-induced cell division in suspension cultures of *Glycine max*. Plant Physiol. **60**: 554-561.

Frankenberger, W.T., Jr., and Arshad, M. 1995. Cytokinins. *In* Phytohormones in soils: microbial production and function. *Edited by* W.T. Frankenberger, Jr. and M. Arshad. Marcel Dekker, Inc., New York. pp. 277-299.

Frankland, B. and Wareing, P.F. 1960. Effect of gibberellic acid on hypocotyl growth of lettuce seedlings. Nature (London) **185**: 225-226.

Fusseder, A., and Ziegler, P. 1988. Metabolism and compartmentalization of dihydrozeatin exogenously supplied to photoautotrophic suspension cultures of *Chenopodium rubrum*. Planta **173**: 104-110.

Gagne, S., Dehbi, L., Le Quere, D., Cayer, F., Morin, J.-L., Lemay R., and Fournier, N. 1993. Increase of greenhouse tomato fruit yields by plant growth-promoting rhizobacteria (PGPR) inoculated into the peat-based growing media. Soil Biol. Biochem. **25**: 269-272.

Galston, A.W., and Sawhney, R.K. 1990. Polyamines in plant physiology. Plant Physiol. **94**: 406-410.

Gamard, P., Bel-Rhlid, R., Labbe, C., Belanger, R.R., and Paulitz, T. 1996. *Pseudomonas fluorescens* 63-28, a PGPR strain producing several antifungal metabolites. *In* Proceedings of the workshop on biological and integrated control of root diseases in soilless cultures. IOBC/WPRS Bull. Dijon, France. pp. 109-114.

Gamard, P., Sauril, F., Benhamou, N., Belanger, R.R., and Paulitz, T. 1997. Novel butyrolactones with antifungal activity produced by *Pseudomonas aureofaciens* strain 63-68. J. Antibiotics **50**: 742-749.

García de Salamone, I.E., Laballos, N., and Monzón de Asconegui, M.A. 1990. Effect of inoculation with *Azospirillum* of wheat cv. Buck Pucara growing in field conditions. *In* Proceedings of the II National Congress of Wheat. AIANBA. Pergamino, Buenos Aires, Argentina. pp. 101-110.

García de Salamone, I.E., Dobereiner, J., Urquiaga, S., and Boddey, R.M. 1996. Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by the ^{15}N isotope dilution technique. Biol. Fertil. Soils **23**: 249-256.

García-Rodriguez, T., Alvarez, C., and Perez-Silva, J. 1984. Indole-3-acetic acid production by cell-free extracts of *Rhizobium trifolii*. Soil Biol. Biochem. **16**: 677-678.

Garland, J.L. 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol. Biochem. **28**: 213-221.

Gawer, M., Laloue, M., Terrine, C., and Guern, J. 1977. Metabolism and biological significance of benzyladenine-7-glucoside. Plant Sci. Lett. **8**: 267-274.

Gillis, M., Döbereiner, J., Pot, B., Goor, M., Falsen, E., Reinhold, B., and Kerster, K. 1991. Taxonomic relationship between *Pseudomonas rubrisulbalbicans* and some clinically isolated (EF group 1) *Herbaspirillum seropedicae*, and *Aquaspirillum autotrophicum*. *In* Nitrogen fixation. *Edited by* M. Pollinelli, R. Materassi, and M. Vicenzini. Kluwer Academic Press, Dordrecht. pp. 292-294.

Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. **41**: 109-117.

Glick, B.R., Jacobson, C.B., Schwarze, M.M.K., and Pasternak, J.J. 1994. Does the enzyme 1-aminocyclopropane-1-carboxylase deaminase play a role in plant growth-promotion by *Pseudomonas putida* GR12-2? *In* Improving plant productivity with rhizophere bacteria. *Edited by* M.H. Ryder, P.M. Stephens and G.D. Bowen. CSIRO, Adelaide, Australia. pp. 150-152.

Gonzalez-Lopez, J., Salmeron, V., Martinez-Toledo, M.V., Ballesteros, F., and Ramos-Cormenzana, A. 1986. Production of auxins, gibberellins and cytokinins by *Azotobacter vinelandi* ATCC 12837 in chemically defined media and dialysed soil media. Soil Biol. Biochem. **18**: 119-120.

Goodwin, P.B., Gollnow, B.I., and Letham, D.S. 1978. Phytohormones and growth correlations. *In* Phytohormones and related compounds: a comprehensive treatise. *Edited by* D.S. Letham, P. Goodwin and T.J.V. Higgings. Elsevier/North Holland, Biomedical Press. New York. pp. 264-314.

Gordon, M.E., Letham, D.S., and Parker, C.W. 1974. The metabolism and translocation of zeatin in intact radish seedlings. Ann. Bot. **38**: 809-835.

Goto, M. 1992. Fundamentals of bacterial plant pathology. Academic Press. New York.

Grayston, S.J., Stephens, J.H., and Nelson, L.M. 1990. Field and greenhouse studies on growth promotion of spring wheat inoculated with co-existent rhizobacteria. *In* Plant growth-promoting rhizobacteria: progress and prospects. *Edited by* C. Keel, B. Koller, and G. Defago. International Union of Biological Sciences, Switzerland. pp. 11-16.

Greene, E. 1980. Cytokinin production by microorganisms. Bot. Rev. **46**: 25-74.

Grimes, H.D., and Mount, M.S. 1984. Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol. Biochem. **16**: 27-30.

Hacker, B., Vunakis, H.V., and Levine, L. 1970. Formation of an antibody with serological specificity for N^6-N^2-isopentenyl adenosine. J. Immunol. **108**: 1726-1732.

Hahn, H., and Bopp, M. 1968. A cytokinin test with high specificity. Planta **83**: 115-121.

Hammerton, R.D., Nicander, B., and Tillberg, E. 1998. Irradiance-induced alterations of growth and cytokinins in *Phaseolus vulgaris* seedlings. J. Plant Growth Regul. **25**: 63-69.

Handelsman, J., and Stabb, E.V. 1996. Biocontrol of soilborne plant pathogens. Plant Cell **8**: 1855-1869.

Hansen, C.E., Meins, F., and Milani, A. 1985. Clonal and physiological variation in cytokinin content of tobacco cell lines differing in cytokinin requirement and capacity for neoplastic growth. Different. **29**: 1-8.

Hansen, C.E., Meins, F., and Aebu, R. 1987. Hormonal regulation of zeatin riboside accumulation by cultured tobacco cells. Planta **172**: 520-526.

Hansen, C.E., Kopperud, C., and Heide, O.M. 1988. Identity of cytokinins in *Begonia* leaves and their variation in relation to photoperiod and temperature. Physiol. Plant. **73**: 387-393.

Hartmann, A., Sing, M., and Klingmuller, W. 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. Can. J. Microbiol. **29**: 916-923.

Hedden, P. 1986. The use of combined gas chromatography-mass spectrometry in the analysis of plant growth substances. *In* Modern methods of plant analysis. *Edited by* H.F. Linskens and J.F. Jackson. Springer-Verlag, Berlin. pp. 1-21.

Heijnen, C.E., Hoh-a-Hin, C.H., and Van Elsas, J.D. 1993. Root colonization by *Pseudomonas fluorescens* introduced into soil amended with bentonite. Soil Biol. Biochem. **25**: 239-246.

Henson, I.E., and Wareing, P.F. 1976. Cytokinins in *Xanthium strumarium* L.: distribution in the plant and production in the root system. J. Exp. Bot. **27**: 1268-1278.

Ho, I. 1986. Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity, and phytohormone production. Can. J. For. Res. **17**: 31-35.

Hoagland, D.R., and Arnon, D.I. 1938. The water-culture method for growing plants without soil. US State Agricultural Experiment Station. Berkeley, California.

Hocart, C.H., and Letham, D.S. 1990. Biosynthesis of cytokinin in germinating seeds of *Zea mays*. J. Exp. Bot. **41**: 1525-1528.

Hong, Y., Pasternak, J. J., and Glick, B. R. 1991. Biological consequences of plasmid transformation of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Can. J. Microbiol. **37**: 796-799.

Horgan, R. 1978. Analytical procedures for cytokinins. *In* Isolation of plant growth substances. *Edited by* J. R. Hillman. Cambridge University Press, Cambridge. pp. 97-114.

Houssa, C., Jacqmard, A., and Bernier, G. 1990. Activation of replicon origins as a possible target for cytokinins in shoot meristems of *Sinapis*. Planta **181**: 324-340.

Howie, W.J., Cook, R.J., and Weller, D.M. 1987. Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppresive to take-all. Phytopathol. **77**: 286-292.

Hubbell, D.H., Tien, T.M., Gaskins, M.H., and Lee, J. 1979. Physiological interaction in the *Azospirillum*-grass root association. *In* CRC associative symbiosis. *Edited by* P.B. Vose, and A.P. Uschel. CRC Press, Boca-Raton. pp. 1-6.

Hussain, A., Arshad, M., Hussain, A., and Hussain, F. 1987. Response of maize (*Zea mays*) to *Azotobacter* inoculation under fertilized and unfertilized conditions. Biol. Fertil. Soils **4**: 73-77.

Hutton, M.J., Van Staden, J., and Davey, J.E. 1982. Cytokinin in germinating seeds of *Phaseolus vulgaris* L. I. Changes in endogenous levels within cotyledons. Ann. Bot. **49**: 685-695.

Ilan, I., and Gepstein, S. 1981. Hormonal regulation of food reserve breakdown in germinating dicotyledonous seeds. Israel. J. Bot. **29**: 193-199.

Iwamura, H., Masuda, N., Koshimizu, K., and Matsubara, S. 1979. Cytokinin-agonistic and antagonistic activities of 4-substituted-2-methylpyrrol[2,3-d]pyrimidines, 7-diazo analogs of cytokinin-active adenine derivatives. Phytochem. **18**: 217-225.

Jacqmard, A., Houssa, C., and Bernier, G. 1994. Regulation of the cell cycle. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 197-215.

Jagnow, G. 1987. Inoculation of cereal crops and forage grasses with N-fixing rhizosphere bacteria: possible causes of success and failure with regard to yield response. A review. Z. Pflanzenernahr Bodenk. **150**: 361-368.

Jameson, P.E. 1994. Cytokinin metabolism and compartmentalization. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 113-128.

Jameson, P.E., and Morris, R.O. 1989. Zeatin-like cytokinins in yeast: detection by immunological methods. J. Plant Physiol. **135**: 385-390.

Jameson, P.E., McWha, J.A., and Wright, G.J. 1982. Cytokinins and changes in their activities during the development of grains of wheat (*Triticum aestivum* L.). J. Plant Physiol. **106**: 27-36.

Jones, R.T., Schreiber, B.M., McNeil, K., and Brenner, M.L. 1990. Hormonal regulation of maize kernel development: the role of cytokinins. Proc. Plant Growth Regul. Soc. Am. **17**: 183-196.

Juhnke, M.E., Mathre, D.E., and Sands, D.C. 1987. Identification and characterization of rhizosphere-competent bacteria of wheat. Appl. Environ. Microbiol. **53**: 2793-2799.

Kaminek, M. 1992. Progress in cytokinin research. TIBTECH. **10**: 159-164.

Kampert, M., Strzelczyk, E., and Pokojska, A. 1975. Production of gibberellin-like substances by bacteria and fungi isolated from roots of pine seedlings. Acta Microbiol. Pol. 7: 157-166.

Kannangara, T., Durley, R.C., and Simpson, G.M. 1978. High performance liquid chromatography analysis of cytokinins in *Sorghum bicolor* leaves. Physiol. Plant. 44: 295-299.

Kapulnik, Y., Gafny, R., and Okon, Y. 1984. Effect of *Azospirillum* spp. inoculation on root development and nitrate uptake in wheat (*Triticum aestivum* cv. Miriam) in hydroponic systems. Can. J. Bot. 63: 627-631.

Kapulnik, Y., Okon, Y., and Henis, Y. 1987. Yield response of spring wheat cultivars (*Triticum aestivum* and *T. turgidum*) to inoculation with *Azospirillum brasilense* under field conditions. Biol. Fertil. Soils 4:27-35.

Karanov, E., Iliev, L., Georgiev, G.T.S., Tsolova, M., Alexieva, V., and Puneva, I. 1992. Physiology and application of phenylurea cytokinins. *In* Progress in plant growth regulation. *Edited by* C.M. Karssen, L.C. Van Loon, and D. Vreugdenhil. Kluwer Academic Publishers, The Netherlands. pp. 842-845.

Karmoker, J.L. and Van Steveninck, R.F.M. 1979. The effect of absicisic acid on the uptake and distribution of ions in intact seedlings of *Phaseolus vulgaris* cv. Redland Pioneer. Physiol. Plant. 45, 453-459.

Keel, C., Koller, B., and Defago C.D. 1990. Plant growth-promoting rhizobacteria: progress and prospects. International Union of Biological Sciences, Switzerland.

Keel, C., and Defago, G. 1997. Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. *In* Multitrophic interactions in terrestial systems. *Edited by* A.C. Gange, and V.K. Brown. Blackwell Science, Oxford, UK. pp. 27-46.

Khammas, K.M., Ageron, E., Grimont, P.A.D., and Kaiser, P. 1989. *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere Soil Res. Microbiol. 140: 679-693.

King, P.J. 1988. Plant hormone mutants. Trends Genet. 4: 157-163.

Klee, H. 1994. Transgenic plants and cytokinin biology. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 289-293.

Klee, H., and Estelle, M. 1991. Molecular genetic approaches to plant hormone biology. Ann. Rev. Plant Physiol. 42: 529-551.

Kloepper, J.W.E. 1993. Plant growth-promoting rhizobacteria as biological agents. *In* Soil microbial ecology. Applications in agricultural and enviromental management. *Edited by* F. B. Metting, Jr. Marcel Dekker. New York. pp. 255-274.

Kloepper, J.W.E., and Schroth, M. N. 1978. Plant growth-promoting rhizobacteria on radishes. *In* Proceedings of the fourth international conference on plant pathogenic bacteria. *Edited by* A. Gibert-Clarey. INRA, Tours. pp. 879-882.

Kloepper, J.W.E., Lifshitz, R., and Schroth, M. N. 1988. *Pseudomonas* inoculants to benefit plant production. ISI Atlas Sci. Anim. Plant Sci. pp. 60-64.

Kloepper, J.W.E., Lifshitz, R., and Zablotowicz, R. M. 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. **7**: 39-44.

Kloepper, J.W.E., Young, S. Lifshitz, R., and Zablotowicz, R.M. 1990. Description of a coryneform plant growth-promoting rhizobacteria (PGPR) strain and its effects on plant development. *In* Plant pathogenic bacteria. *Edited by* A. Z. Klement. Akademia: Kiado, Budapest. pp. 613-618.

Kloepper, J.W.E., Schippers, B., and Bakker, P.A.M. 1992. Proposed elimination of the term endorhizosphere. Phytopathol. **82**: 726-727.

Kohler, K.H., Opitz, C., and Fritsch, G. 1987. Physiology and biochemistry of the *Amaranthus* cytokinin bioassay and its applications. Biol. Plant. **29**: 10-16.

Kraigher, H., Grayling, A., Wang, T.L., and Hanke, D.E. 1991. Cytokinin production by two ectomycorrizal fungi in liquid culture. Phytochem. **30**: 2249-2253.

Kucey, R.M.N. 1988. Plant growth-altering effects of *Azospirillum brasilense* and *Bacillus* C-11-25 on two wheat cultivars. J. Appl. Bacteriol. **64**: 187-196.

Kumar, P.K.R., and Lonsane, B.K. 1989. Microbial production of gibberellins: state of the art. Adv. Appl. Microbiol. **34**: 29-139.

Kuraishi, S., and Okamura, F.S. 1956. The effect of kinetin on leaf growth. Bot. Mag. **69**: 817-823.

Laloue, M., and Fox, J.E. 1989. Cytokinin oxidase from wheat: partial purification and general properties. Plant Physiol. **90**: 899-906.

Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J.M., and Alabouvette, C. 1995. Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicum esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. Appl. Environ. Microbiol. **61**: 1004-1012.

Leopold, A.C., and Kawase, M. 1964. Benzyladenine effects on bean leaf growth and senescence. Am. J. Bot. **51**: 294-299.

Letham, D.S. 1963. Zeatin, a factor inducing cell division from *Zea mays*. Life Sci. **8**: 569-573.

Letham, D.S. 1967. Regulators of cell division in plant tissues. V. A comparison of the activities of zeatin and other cytokinins in five bioassays. Planta **74**: 228-234.

Letham, D.S. 1971. Regulators of cell division in plant tissues. XII. A cytokinin bioassay using excised radish cotyledons. Physiol. Plant. **25**: 391-396.

Letham, D.S. 1978. Cytokinins. *In* Phytohormones and related compounds: a comprehensive treatise. *Edited by* D.S. Letham, P. Goodwin, and T.J.V. Higgings. Elsevier/North Holland, Biomedical Press, New York. pp. 205-263.

Letham, D.S. 1994. Cytokinins as phytohormones: sites of biosynthesis, translocation, and function of translocated cytokinins. *In* Cytokinins: chemistry, activity and function. *Edited by* D. W. S. Mok and M. C. Mok. CRC Press Inc., Boca-Raton. pp. 57-80.

Letham, D.S., and Gollnow, B.I. 1985. Regulation of cell division in plant tissues. XXX.Cytokinin metabolism in relation to radish cotyledon expansion and senescence. J. Plant Growth Regul. **2**: 103-115.

Letham, D.S., and Palni, L.M.S. 1983. The biosynthesis and metabolism of cytokinins. Ann. Rev. Plant Physiol. **34**: 163-197.

Letham, D.S., Shannon, J.S., and McDonald, I.R. 1964. The structure of zeatin, a factor inducing cell division. Proc. Chem. Soc. Lond. 230-231.

Letham, D.S., Palni, L.M.S., Tao, G.Q., Gollnow, B.I., and Bates, C.M. 1983. Regulators of cell division in plant tissues XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J. Plant Growth Regul. **2**: 103-115.

Letham, D.S., Parker, C.W., Zhang, R., Singh, S., Upadhyaya, M.N., Dart, P.J., and Palni, L.M.S. 1990. Xylem-translocated cytokinins: metabolism and function. *In* Plant growth substances. *Edited by* R.P. Pharis and S.B. Rood.

Springer-Verlag, Berlin. pp. 275-281.

Lieberman, M. 1979. Biosynthesis and action of ethylene. Ann. Rev. Plant Physiol. **30**: 533-545.

Lifshitz, R., Kloepper, J.W.E., Scher, F.M., Tipping, E.M., and Laliberte, M. 1986. Nitrogen-fixing pseudomonads isolated from roots of plants grown in the Canadian high arctic. Appl. Environ. Microbiol. **51**: 251-255.

Lifshitz, R., Kloepper, J.W.E., Kozlowksi, M., Simonson, C., Carlson, J., Tipping, E.M., and Zaleska, I. 1987. Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can. J. Microbiol. **33**: 390-395.

Linsmaier, E.M., and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol Plant. **18**: 100-127.

Lips, S.H., and Roth-Bejerano, N. 1969. Light and hormones: interchangeability in the induction of nitrate reductase. Science **166**: 109-111.

Longo, G.P., Longo, C.P., Rossi, G., Vitale, A., and Predetti, M. 1978. Variations in carbohydrates and in osmotic potential of watermelon cotyledons treated with benzyladenine. Plant Sci. Lett. **12**: 179-185.

Loper, J.E., Nowak-Thompson, B., Whistler, C.A., Hagen, M.J., Corbell, N.A., Henkels, M.D., and Stockwell, V.O. 1997. Biological control mediated by antifungal metabolite production and resource competition: an overview. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 73-81.

Louis, J.-P., Augur, C., and Teller, G. 1990. Cytokinins and differentiation processes in *Mercurialis annua*. Plant Physiol. **94**: 1535-1441.

Lu, J.L., Ertl, J.R., and Chen, C.M. 1992. Transcriptional regulation of nitrate reductase mRNA levels by cytokinin-abscisic acid interactions in etiolated barley leaves. Plant Physiol. **98**: 1255-1260.

Lugtenberg, B.J.J., Weger de, L.A., and Schippers, B. 1994. Bacterization to protect seed and rhizosphere against disease. *In* Seed treatments: progress and prospects. *Edited by* T. Martin. Crop Protection Council, Farnham, UK. pp. 293-302.

Lur, H.S., and Setter, T.L. 1993. Role of auxin in maize endosperm development. Plant Physiol. **103**: 273-280.

Lynch, J.M. 1990. The Rhizosphere. John Wiley, Chichester, U.K.

Lynch, J.M. 1994. The rhizosphere - form and function. Appl. Soil Ecol. **1**: 193-198.

Lynch, J.M., and Whipps, J.M. 1990. Substrate flow in the rhizosphere. Plant Soil **129**: 1-10.

MacDonald, E.M.S., and Morris, R.O. 1985. Isolation of cytokinins by immunoaffinity chromatography and analysis of high-performance liquid chromatography. Methods Enzymol. **110**: 347-358.

MacDonald, E.M.S., Akiyoshi, D.E., and Morris, R.O. 1981. Combined high-performance liquid chromatography-radioimmunoassay for cytokinins. J. Chromatogr. **214**: 101-109.

MacDonald, E.M.S., Powell, G.K., Regier, D.A., Glass, N.L., Roberto, F., Kosuge, T., and Morris, R.O. 1986. Secretion of zeatin and ribosylzeatin, and ribosyl-1-methylzeatin by *Pseudomonas savastanoi*. Plant Physiol. **82**: 742-747.

MacLeod, R.D. 1968. Changes in the mitotic cycle in lateral root meristems of *Vicia faba* following kinetin treatment. Chromosoma **24**: 177-183.

Magalhaes, F.M.M., Baldani, J.I., Souto, S.M., Kuykendall, J.R., and Döbereiner, J. 1983. A new acid-tolerant *Azospirillum* species. An. Acad. Bras. Cien. **55**: 417-430.

Maki, H., Ando, S., Kodama, H., and Komamine, A. 1991. Polyamines and the cell cycle of *Catharanthus roseus*. Plant Physiol. **96**: 1008-1014.

Mapelli, S., and Lombardi, L. 1982. A comparative auxin and cytokinin study in normal and to-2 tomato plants. Plant Cell Physiol. **23**: 751-757.

Mariano, R.L.R., Michereff, E., Silveira, E.B., Assis, S.M.P., and Reis, A. 1997. Plant growth-promoting rhizobacteria in Brazil. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino, Nakanishi Printing, Sapporo, Japan. pp. 22-29.

Marx, D.H. 1981. Variability in ectomycorrhizal development and plant growth among isolates of *Pisolithus tinctorius*. Can. J. Microbiol. **23**: 217-223.

Marziani Longo, G.P., Rossi, G., and Longo, C.P. 1991. Endogenous levels of three cytokinins in attached and excised watermelon cotyledons. Physiol. Plant. **83**: 647-653.

Matsubara, S. 1980. Structure-activity relationships of cytokinins. Phytochem. **19**: 2239-2245.

McGaw, B.A., and Horgan, R. 1983. Cytokinin oxidase from *Zea mays* kernels and *Vicia rosea* crown-gall tissue. Planta **159**: 30-37.

McGaw, B.A., and Horgan, R. 1985. Cytokinin metabolism and the control of cytokinin activity. Biol. Plant. **27**: 180-186.

McGaw, B.A., Heald, J.K., and Horgan, R. 1984. Dihydrozeatin metabolism in radish seedlings. Phytochem. **23**: 1373-1377.

McGaw, B.A., Horgan, R., and Heald, J.K. 1985. Cytokinin metabolism and the modulation of cytokinin activity. Phytochem. **24**: 9-15.

Meyer, Y., and Cooke, R. 1979. Time course of hormonal control of the mitosis in tobacco mesophyll protoplast cultivated in vitro. Planta **147**: 181-187.

Michael, G., and Seiler-Kelbitsch, H. 1972. Cytokinin content and kernel size of barley grains as affected by environmental and genetic factors. Crop Sci. **12**: 162-165.

Miller, C.O. 1958. The relationship of the kinetin and red-light promotions of lettuce seed germination. Plant Physiol. **33**: 115-121.

Miller, C.O. 1961. Kinetin and related compounds in plant growth. Adv. Rev. Plant Physiol. **12**: 395-399.

Miller, C.O. 1963. Kinetin and kinetin-like compounds. Mod. Methods Plant Anal. **6**: 194-202.

Miller, C.O. 1968. Naturally-occurring cytokinins. *In* Biochemistry and physiology of plant growth substances. *Edited by* F. Wightman and F. Setterfield. Runge Press, Ottawa. pp. 33-45.

Miller, C.O., Skoog, F., Okomura, F.S. von Saltza M.H., and Strong, F.M. 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. **78**: 1345-1350.

Miller, H.J., Liljeroth, E., Henken, G., and Van Veen, J.A. 1989. Fluctuations in the fluorescent pseudomonad and actinomycete populations of rhizosphere and rhizoplane during the growth of spring wheat. Can. J. Microbiol. **36**: 254-258.

Milstone, D.S., Vold, B.S., Glitz, D.G., and Shutt, N. 1978. Antibodies to N^6-(N^2-isopentenyl) adenosine and its nucleotide: interaction with purified tRNAs and with bases, nucleosides and nucleotides of the isopentenyladenosine

family. Nucleic Acids Res. 5: 3439-3445.

Milus, E.A., and Rothrock, C.S. 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. Can. J. Microbiol. 39: 335-341.

Miralles, D.J., and Slafer, G.A. 1991. A simple model for non-destructive estimate of leaf area in wheat. Cereal Res. Comm. 19: 439-444.

Mok, M.C. 1994. Cytokinins and plant development: an overview. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 155-166.

Mok, D.W.S., and Martin, R. 1994. Cytokinin metabolic enzymes. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 129-137.

Mok, M.C., Mok, D.W.S., Armstrong, D.J., Rabakoarihanta, A., and Kim, S.G. 1980. Cytokinin autonomy in tissue cultures of *Phaseolus*: a genotype-specific and heritable trait. Genetics 94: 675-686.

Mok, M.C., Mok, D.W.S., Marsden, K.E., and Shaw, G. 1987a. The biological activity and metabolism of a novel cytokinin metabolite, O-xylosylzeatin, in callus tissue of *Phaseolus vulgaris* and *P. lunatus*. J. Plant Physiol. 130: 423-431.

Mok, M.C., Mok, D.W.S., Turner, J.E., and Mujer, C.V. 1987b. Biological and biochemical effects of cytokinin active phenylurea derivatives in tissue culture systems. Hortsci. 22: 1194-1200.

Molina, R. 1979. Ectomycorrhizal inoculation on containerized Douglas-fir and lodgepole pine seedlings with six isolates of *Pisolithus tinctorius*. Forest Sci. 25: 585-590.

Morgenstern, E., and Okon, Y. 1987. Promotion of plant growth and NO^{3-} and Rb^{+} uptake in *Sorghum bicolor* x *Sorghum sudanense* inoculated with *Azospirillum brasilense* Cd. Arid Soil Res. Rehabil. 1: 211-217.

Morohashi, Y. 1982. Control of development of aminolytic and proteolytic activities in cotyledons of germinating black gram seeds. Physiol. Plant. 56: 189-195.

Morris, R.O. 1986. Genes specifying auxin and cytokinin biosynthesis in phytopathogens. Ann. Rev. Plant Physiol. 37: 509-538.

Morris, R.O., Jameson, P.E., Laloue, M. and Morris, J.W. 1991. Rapid

identification of cytokinins by an immunological method. Plant Physiol. **65**: 1156-1161.

Morris, R.O., Blevius, D.G., Dietrich, J.T., Durley, R.C., Gelvin, S.B., Gray, J., Hommes, N.G., Kaminek, M., Mathews, L.J., Meilan, R., T.M., R., and Saavedra-Soto, L. 1993. Cytokinins in plant pathogenic bacteria and developing cereal grains. Aust. J. Plant Physiol. **20**: 621-637.

Mothes, K., and Engelbrecht, L. 1961. Kinetin-induced directed transport of substances in excised leaves in the dark. Phytochem. **1**: 58-64.

Motyka, V., and Kaminek, M. 1992. Characterization of cytokinin oxidase from tobacco and poplar callus cultures. *In* Physiology and biochemistry of cytokinins in plants. *Edited by* M. Kaminek, D.W.S. Mok, and E. Zazimalova. Academic Press, The Hague. pp. 33-39.

Muller, M., Diegele, C., and Ziegler, H. 1988. Hormonal interactions in the rhizosphere of maize (*Zea mays*) and their effects on plant development. Z. Pflanzenernahr. Bodenk. **152**: 247-254.

Murai, N. 1994. Cytokinin biosynthesis in tRNA and cytokinin incorporation into plant RNA. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 87-98.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Murty, M.G., and Ladha, J.K. 1988. Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions. Plant Soil **108**: 281-285.

Musgrave, M.E. 1994. Cytokinin and oxidative processes. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 167-178.

Nandi, S.K., Palni, L.M.S., Letham, D.S., and Knypl, J.S. 1988. The biosynthesis of cytokinins in germinating lupin seed. J. Exp. Bot. **39**: 1649-1665.

Nandi, S.K., Palni, L.M.S., and Parker, C.W. 1990. Dynamics of endogenous cytokinins during the growth cycle of a hormone-autotrophic genetic tumor line of tobacco. Plant Physiol. **94**: 1084-1089.

Navratil, S., and Rochon, C.G. 1981. Enhanced root and shoot development of poplar cutting induced by *Pisolithus* inoculum. Can. J. For. Res. **11**: 844-848.

Neuman, D.S., Rood, S.B., and Smith, B.A. 1990. Does cytokinin transport from root-shoot in the xylem sap regulate leaf responses to root hypoxia? J. Exp. Bot. **41**: 1325-1331.

Newcomb, W., Syono, K., and Torrey, J.G. 1977. Development of an ineffective pea root nodule: morphogenesis, fine structure and cytokinin biosynthesis. Can. J. Bot. **55**: 1891-1907.

Nielsen, T. H., and Ulvskov, P. 1992. Cytokinins and leaf development in sweet pepper (*Capsicum annum*) II. Sink metabolism in relation to cytokinin-promoted leaf expansion. Planta **188**: 70-76.

Nieto, K.F., and Frankenberger, W.T., Jr. 1989. Biosynthesis of cytokinins by *Azotobacter chroococcum*. Soil Biol. Biochem. **21**: 967-972.

Nijhuis, E.H., Maat, M.J., Zeegers, I.W., Waalwijk, C., and Van Veen, J.A. 1993. Selection of bacteria suitable for introduction into the rhizosphere. Soil Biol. Biochem. **25**: 885-895.

Nishinari, N., and Syono, K. 1986. Induction of cell division synchrony and variation of cytokinin contents through the cell cycle. Plant Cell. Physiol. **27**: 147-153.

Nooden, L.D., and Letham, D.S. 1993. Cytokinin metabolism and signalling in the soybean plant. Aust. J. Plant Physiol. **20**: 639-645.

Nooden, L.D., Guiamet, J.J., Singh, S., Letham, D.S., Tsuji, J., and Schneider, M.J. 1990a. Hormonal control of senescence. *In* Plant growth substances. *Edited by* R.P. Pharis and S.V. Rood. Springer-Verlag, Berlin. pp. 537-546.

Nooden, L.D., Singh, S., and Letham, D.S. 1990b. Correlation of xylem sap cytokinin levels with monocarpic senescence in soybean. Plant Physiol. **93**: 33-40.

Norris, R.F. 1976. Expansion of radish cotyledons: an interaction between NaCl and cytokinins. Plant Sci. Lett. **6**: 63-69.

Ogoshi, A., Kobayashi, K., Homma, Y., Kodama, F., Kondo, N., and Akino, S. 1997. Plant growth-promoting rhizobacteria. Present, status and future prospects. Nakanishi Printing, Sapporo, Japan.

Okamoto, T., Shudo, K., Takahashi, S., Yatsunani, T., Isogai, Y., and Yamada, K. 1978. Shoot formation of tobacco callus by various cytokinin-active ureas and pyrimidines. Chem. Pharm. Bull. **26**: 3250-3256.

Okon, Y., and Labandera, C.A. 1994. Agronomic applications of *Azospirillum*.

In Improving plant productivity with rhizophere bacteria. *Edited by* M.H. Ryder, P.M. Stephens, and G.D. Bowen. CSIRO, Adelaide, Australia, pp. 274-278.

Olivares, F.L., Baldani, V.L.D., Veronica, V.M., Baldani, J.I., and Döbereiner, J. 1996. Occurence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stem, and leaves, predominantly of Gramineae. Biol. Fertil. Soils **21**: 197-200.

Ordas, R.J., Fernandez, B., and Rodriguez, R. 1992. Benzyladenine-controlled protein synthesis and growth in apple cell suspensions. Physiol. Plant. **84**: 229-235.

Osborne, D.J., and McCalla, D.R. 1961. Rapid bioassay for kinetin and kinins using senescing leaf tissue. Plant Physiol. **36**: 219-225.

O'Sullivan, D.J., Stephens, P.M., and O'Gara, F. 1991. Extracellular protease production by fluorescent *Pseudomonas* spp and colonization of sugarbeet roots and soil. Soil Biol. Biochem. **23**: 623-627.

O'Sullivan, D.J., and O'Gara, F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56**: 662-676.

Palni, L.M.S., Tay, S.A.B., and MacLeod, J.K. 1986. GC-MS methods for cytokinins and metabolites. *In* Modern methods of plant analysis. *Edited by* H.F. Linskens, and J.F. Jackson. Springer-Verlag, Berlin. pp. 214-253.

Palni, L.M.S., Burch, L., and Horgan, R. 1988. The effect of auxin concentration on cytokinin stability and metabolism. Planta **174**: 231-237.

Palni, L.M.S., Nandi, S.K., Singh, S., and Letham, D.S. 1990. An overview of cytokinin biosynthesis. *In* Plant growth substances. *Edited by* R.P. Pharis and S.V. Rood. Springer-Berlag, Berlin. pp. 258-266.

Parker, C.W., and Letham, D.S. 1973. Regulators of cell division in plant tissues. XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. Planta **114**: 199-218.

Pastula, C.L., Neisser, C.D., Funk, L.M., Pedersen, E.A., and Reddy, M.S. 1996. Preliminary investigations on the modes of action of biological control agents: *Burkholderia cepacia* strains Ral-3 and Ral-3R and *Pseudomonas aureofaciens* strain 63-28. *In* Annual Meeting of The 67th Canadian Phytopathological Society. *Edited by* The Canadian Phytopathological Society. Saskatoon, SK, Canada, Abstr. 10.

Patrick, J.W. 1987. Are the hormones involved in assimilate transport? *In* Hormone action in plant development: a critical appraisal. *Edited by* G.V. Hoad, J.R. Lenton, M.B. Jackson, and R.K. Atkin. Butterworths Co. Ltd., Long Ashton,

UK. pp. 175-188.

Paul, E.A., and Clark, F.E. 1989. Soil microbiology and biochemistry. Academic Press, Inc., San Diego.

Pedersen, E.A., and Reddy, M.S. 1996. Potential of *Burkholderia cepacia* as a biological control agent for multiple crops against damping-off and root rot pathogens. In Advances in biological control of plant diseases. *Edited by* T. Wenhua, R.J. Cook, and A. Rovira. China Agricultural University Press, Beijing, China. pp. 89-93.

Penner, D., and Ashton, F.M. 1967. Hormonal control of proteinase activity in squash cotyledons. Plant Physiol. **42**: 791-795.

Pfosser, M., Konigshofer, H., and Kandeler, R. 1990. Free, conjugated and bound polyamines during the cell cycle of synchronized cell suspension cultures of *Nicotiana tabacum*. J. Plant Physiol. **135**: 574-581.

Phillips, D.A., and Torrey, J.G. 1972. Studies on cytokinin production by *Rhizobium*. Plant Physiol. **49**: 11-15.

Pierson, E.A., and Weller, D.M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathol. **84**: 940-947.

Piga, P.M., Belanger, R.R., Paulitz, T.C., and Benhamou, N. 1997. Increased resistance to *Fusarium oxysporum* f. sp. radicis-lycopersici in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiol. Mol. Plant Pathol. **50**: 301-320.

Pilate, G., Sossountzov, L., and Miginiac, E. 1989. Hormone levels and apical dominance in the aquatic fern *Marsilea drummondii*. A. Br. Plant Physiol **90**: 907-913.

Pimentel, J.P., Olivares, F.L., Pitard, R.M., Urquiaga, S., Akiba, F., and Döbereiner, J. 1991. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum*. Plant Soil **137**: 1-65.

Pino, E., Martin, L., Guerra, H., Nicolas, G., and Villalobos, N. 1991. Effect of dihydrozeatin on the mobilization of protein reserves in protein bodies during germination of chick-pea seeds. J. Plant Physiol. **137**: 425-431.

Polonenko, D.R., Scher, F.M., Kloepper, J.W.E., Singleton, C.A., Laliberte, M., and Zaleska, I. 1987. Effects of root colonizing bacteria on nodulation of soybean roots by mutants affected in antibiosis. Can. J. Microbiol. **33**: 498-503.

Primrose, S.B. 1979. A review. Ethylene and agriculture: the role of microbes. J. Appl. Microbiol. **46**: 1-25.

Prinsen, E., Redig, P., Van Dongen, W., Esmans, E.L., and Van Onckelen, H. 1995. Quantitative analysis of cytokinins by electrospray tandem mass spectrometry. Rapid Comm. Mass. Spec. **9**: 948-953.

Puppo, A., and Rigaud, J. 1978. Cytokinins and morphological aspects of French-bean roots in the present of *Rhizobium*. Physiol. Plant. **42**: 202-206.

Radin, J.W., and Loomis, R.S. 1974. Polar transport of kinetin in tissues of radish. Plant Physiol. **53**: 348-351.

Raupach, G.S., and Kloepper, J.M. 1997. Integrated pest management of multiple cucumber pathogens through PGPR-mediated induced systemic resistance. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 281-284.

Reddy, M.S., Campbell, S.E., Young, S.E., and Brown, G. 1990. Greenhouse evaluation of rhizobacteria for the suppression of crown and root rot of tomato caused by *Fusarium oxysporum* F.S.P. *Radicis-Lycopersici*. *In* Plant growth-promoting rhizobacteria: progress and prospects. *Edited by* C. Keel, B. Koller, and G. Defago. International Union of Biological Sciences, Switzerland. pp. 39-44.

Reddy, M.S., Hynes, R.K., and Lazarovits, G. 1994. Relationship between in vitro growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria. Can. J. Microbiol. **40**: 113-119.

Reddy, M.S., Funk, L.M., Covert, D.C., He, D.N., and Pedersen, E.A. 1997. Microbial inoculant for sustainable forests. J. Sust. Forest. **5**: 293-306.

Reid, J.B. 1993. Plant hormone mutants. J. Plant Growth Regul. **12**: 207-226.

Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis M., Kersters, K., Thielemans, D., and Deley, J. 1987. *Azospirillum halopraeferans* sp. nov. a nitrogen fixing organism associated with roots of Kallar grass (*Leptochloa fusca*). Int. J. Syst. Bacteriol. **37**: 43-51.

Revilla, M.E., Martin, L., Nicolas, G., Legaz, M.E., and Villalobos, N. 1988. Effects of high temperature on the variation and transport of endogenous cytokinins during the germination of chickpea seeds. J. Plant Physiol. **132**: 223-229.

Reynolds, J.F. 1987. Chemical regulation in tissues: an overview. Hortsci. **22**: 1192-1201.

Richmond, A.E., and Lang, A. 1957. Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science **125**: 650-562.

Rossi, W., Grappelli, A., and Pietrosanti, W. 1984. Phytohormones in soil after atrazine application. Folia Microbiol. **29**: 325-329.

Rovira, A. D. 1991. Rhizosphere research: 85 years of progress and frustration. In The rhizosphere and plant growth. *Edited by* L. E. Keister and P. B. Cregan. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 3-13.

Rovira, A. D., Foster, R.C., and Martín, J.K. 1979. Note on terminology: origin, nature and nomenclature of the organic materials in the rhizosphere. *In* The soil root interface. *Edited by* J.L. Harley, and R. Scott Russell. Academic Press, New York. pp. 1-4.

Rubery, P.H. 1987. Manipulation of hormone transport in physiological and development studies. *In* Hormone action in plant development: a critical appraisal. *Edited by* G.V. Hoad, J.R. Lenton, M.B. Jackson, and R.K. Atkin. Butterworths Co. Ltd., Long Ashton, UK. pp. 161-174.

Ryder, M.H., Stephens, P.M., and Bowen, G.D. 1994. Improving plant productivity with rhizosphere bacteria. Proceedings of the Third International Workshop on Plant Growth Promoting Rhizobacteria. CSIRO, Adelaide, Australia.

Ryder, M.H., and McClure, N.C. 1997. Antibiosis in relation to other mechanisms in biocontrol by rhizobacteria. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 65-72.

Saavedra-Soto, L.A., Durley, R.C., Trione, E.J., and Morris, R.O. 1988. Identification of cytokinins in young wheat spikes (*Triticum aestivum* cv. Chinese Spring). J. Plant Growth Regul. **7**: 169-175.

Sachs, R.M. 1965. Stem Elongation. Ann. Rev. Plant. Physiol. **16**: 73-84.

Saha, S., Nagar, P.K., and Sircar, P.K. 1986. Cytokinin concentration gradient in the developing grains and upper leaves of rice (*Oryza sativa*), during grain filling. Can. J. Bot. **64**: 2068-2072.

Salisbury, F.B., and Ross, C.W. 1992. Plant Physiology. Wadsworth Publishing Company, Belmont, California.

Sands, D.C., and Rovira, A.D. 1970. Isolation of fluorescent pseudomonads with a selective medium. *Pseudomonas fluorescens* biotype G, the dominant fluorescent pseudomonad in South Australian soil and wheat rhizospheres. J. Appl. Bacteriol. **34**: 261-275.

Sarig, S., Blum, A., and Okon, Y. 1988. Improvement of the water status and yield of field-grown grain sorghum (*Sorghum bicolor*) by inoculation with *Azospirillum brasilense*. J. Agric. Sci. **110**: 271-277.

Sasse, J.M. 1991. Brassinosterioids: are they endogenous plant hormones? Plant Growth Regul. Soc. Am. **19**: 1-18.

Saunders, M.J. 1994. Cytokinin and signal transduction. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 243-254.

Scher, F.M., Ziegle, J.S., and Kloepper, J.W.E. 1984. A method for assessing the root-colonizing capacity of bacteria on maize. Can. J. Microbiol. **30**: 151-157.

Scher, F.M., Kloepper, J.W.E., and Singleton, C.A. 1985. Chemotaxis of fluorescent *Pseudomonas* spp to soybean seed exudates *in vitro* and in soil. Can. J. Microbiol. **31**: 570-574.

Schippers, B., Bakker, A.W., and Bakker, P.A.H.M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Ann. Rev. Phytopathol. **25**: 339-358.

Schroth, M.N., and Hancock, J.G. 1982. Disease-suppressive soil and root-colonizing bacteria. Science **216**: 1376-1381.

Sequeira, L. 1973. Hormone metabolism in diseased plants. Ann. Rev. Plant Physiol. **24**: 353-380.

Shanahan, P., O'Sullivan, D.J., Simpson, P., Glennon, J.D., and O'Gara, F. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl. Environ. Microbiol. **58**: 353-358.

Shaw, G. 1994. Chemistry of adenine cytokinins. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 15-34.

Shaw, G., and Wilson, D. V. 1964. A synthesis of zeatin. Proc. Chem. Soc. 230-236.

Shaw, G., Smallwood, B., and Steward, F.C. 1971. The structure and physiological activity of some N^6-substituted adenines. Phytochem. **10**: 2329-2335.

Shinshi, H., Mohnen, D., and Meins, F.J. 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitanse mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. **84**: 89-100.

Shudo, K. 1994. Chemistry of phenylurea cytokinins. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 35-42.

Simmons, C.R., Litts, J.C., Huang, N., and Rodriguez, R.L. 1992. Structure of a rice β-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. Plant Mol. Biol. **18**: 33-40.

Simpson, G.M. 1990. Seed dormancy in grasses. Cambridge University Press, New York.

Singh, S., Letham, D. S., Zhang, X., and Palni, L. M. S. 1992. Cytokinin biochemistry in relation to leaf senescence. VI. Effects of nitrogenous nutrients on cytokinin levels and senescence of tobacco leaves. Physiol. Plant. **84**: 262-274.

Skoog, F. and Armstrong, D. J. 1970. Cytokinins. Ann. Rev. Plant Physiol. **21**: 359-361.

Skoog, F., and Miller, C.O. 1957. Chemical regulation of organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. **15**: 118-131.

Skoog, F., Strong, F.M., and Miller, C.O. 1965. Cytokinins. Science. **148**: 532-533

Smith, A.R., and Van Staden, J. 1978. Changes in endogenous cytokinin levels in kernels of *Zea mays* L. during imbibition and germination. J. Exp. Bot. **29**: 1067-1075.

Soby, S., and Bergman, K. 1983. Motility and chemotaxis of *Rhizobium meliloti* in soil. Appl. Environ. Microbiol. **46**: 995-998.

Soejima, H. Sugiyama, T. Ishihara, K. 1992. Changes in cytokinin activities and mass spectrometric analysis of cytokinins in root exudates of rice plant (*Oryza sativa* L.). Plant Physiol. **100**: 1724-1729.

Sossountzov, L., Maldiney, R., Sotta, B., Sabbagh, I., Habricot, Y., Bonnet, M., and Miginiac, E. 1989. Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant. Planta **175**: 291-297.

Sprinzl, M., Dank, N., Nock, S., and Schon, A. 1991. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. **19**: 2127-2131.

Stahl, E. 1969. Thin-layer chromatography, A Laboratory Handbook. Springer-Verlag, Berlin.

Stenlid, G. 1982. Cytokinins as inhibitors of root growth. Physiol. Plant. **56**: 500-506.

Stevenson, F.J. 1986. Cycles of soil: carbon, nitrogen, phosphorous, sulfur and micronutrients. Wiley & Sons, London.

Strzelczyk, E., and Potojska-Burdziej, A. 1984. Production of auxins and gibberellin-like substances by mycorrhizal fungi, bacteria, and actinomycetes isolated from soil and the mycorrhizosphere of pine. (*Pinus silvestris* L.). Plant Soil **81**: 185-194.

Summons, R. E., Entsch, B., Parker, C. W., and Letham, D. S. 1979. Mass spectrometric analysis of cytokinins in plant tissues. III. Quantification of cytokinin glycoside complex of lupin pods by stable isotope dilution. FEBS Lett. **107**: 21-27.

Sundaran, S., and Klucas, R.V. 1988. Characterization of azospirilla isolated from seeds and roots of turf grass. Can. J. Microbiol. **34**: 212-217.

Suslow, T.V. 1982. Role of root-colonizing bacteria in plant growth. *In* Phytopathogenic prokaryotes. *Edited by* M. S. Mount and G. S. Lacy. Academic Press, New York. pp. 187-223.

Tabor, C. W., and Tabor, H. 1984. Polyamines. Ann. Rev. Biochem. **53**: 749-755.

Takagi, M., Yokota, T., Murofushi, N., Saka, H., and Takahashi, N. 1989. Quantitative changes of free base, riboside, ribotide and glucoside cytokinins in developing rice grains. Plant Growth Regul. **8**: 349-364.

Takahashi, S., Shudo, K., Okamoto, T., Yamada, K., and Isogai, Y. 1978. Cytokinin activity of N-phenyl-N'-(4-pyridyl)urea derivatives. Phytochem. **17**: 1201-1207.

Taller, B.J., and Wong, T.Y. 1989. Cytokinins in *Azotobacter vinelandi*. Appl. Environ. Microbiol. **55**: 266-267.

Taller, B.J. 1994. Distribution, biosynthesis, and function of cytokinins in t-RNA. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 101-112.

Tardieu, F., Zhang, J., and Davies, W.J. 1992. What information is conveyed by an ABA signal from maize roots in drying field soils? Plant Cell Environ. **15**: 185-191.

Taylor, J.S., Thompson, B., Pate, J.S., Atkins, C.A., and Pharis, R.P. 1990. Cytokinins in the phloem sap of white lupin (*Lupinus albus* L.). Plant Physiol. **94**: 1714-1720.

Teller, G. 1994. Gas chromatography-mass spectrometric and related methods for the analysis of cytokinins. *In* Cytokinins: chemistry, activity and function. *Edited by* D.W.S. Mok and M.C. Mok. CRC Press Inc., Boca-Raton. pp. 317-323.

Thimann, K. V. 1980. Senescence of leaves. *In* Senescence in plants. *Edited by* K.V. Thimann. CRC Press, Boca-Raton. pp. 85-97.

Thimann, K.V., and Sachs, T. 1966. The role of cytokinins in the fasciation disease caused by *Corynebacterium fasciens*. Am. J. Bot. **53**: 731-742.

Thomas, T.H. 1992. Cytokinin involvement in the control of seed dormancy. *In* Physiology and biochemistry of cytokinins in plants. *Edited by* M. Kaminek, D.W.S. Mok, and E. Zazimalova. SPB Academic Publishers, The Hague. pp. 416-423.

Tien, T. M., Gasking, M. H., and Hubell, D. H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanus* L.). Appl. Environ. Microbiol. **37**: 1016-1026.

Timmusk, S., Nicander, B., Granhall, U., and Tillberg, E. 1999. Cytokinin production by *Paenobacillus polymyxa*. Soil Biol. Biochem. **31**: 1847-1852.

Trewavas, A.J. 1985. Growth substances, calcium, and the regulation of cell division. *In* The cell division cycle in plants. *Edited by* J.A. Bryant and D. Francis. Cambridge University Press, Cambridge. pp. 133-145.

Trewavas, A.J. 1987. Sensitivity and sensory adaptation in growth substances responses. *In* Hormone action in plant development : a critical appraisal. *Edited by* G.V. Hoad, J.R. Lenton, M.B. Jackson, and R.K. Atkin. Butterworths Co. Ltd., Long Ashton, UK. pp. 19-38.

Trewavas, A.J. 1992. Growth substances in context: a decade of sensitivity. Biochem. Soc. Transact. **20**: 102-108.

Turner, J.E., Mok, M.C., and Mok, D.W.S. 1985. Zeatin metabolism in fruits of *Phaseolus*: comparison between embryos, seedcoat, and pod tissues. Plant Physiol. **79**: 321-322.

Turner, J.T., and Blackman, P.A. 1991. Factors relating to peanut yield increases following *Bacillus subtilis* seed treatment. Plant Dis. **75**: 347-353.

Tuzun, S., and Kloepper, J.W.E. 1995. Practical application and implementation of induced resistance. *In* Induced resistance to disease in plants. *Edited by* R. Hammershchmidt and J. Kuc. Kluwer Academic Press, Boston. pp. 152-168.

Ulstov, P., Nielsen, T.H., Seiden, P., and Marcussen, J. 1992. Cytokinins and leaf development on sweet pepper (*Capsicum annuum* L.). I. Spatial distribution of endogenous cytokinin in relation to leaf growth. Planta **188**: 70-76.

Upadhyaya, N.M., Parker, C.W., Letham, D.S., Scott, K.F., and Dart, P.J. 1991. Evidence for cytokinin involvement in *Rhizobium* (IC3342)-induced leaf curl syndrome of pigeonpea (*Cajanus cajan* Millsp.). Plant Physiol. **95**: 1019-1025.

Vagnerova, K., Macura, J., and Catska, V. 1960. Rhizosphere microflora of wheat. I. Composition and properties of bacterial flora during the first stages of growth. Folia Microbiol. **5**: 298-310.

Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. 1997. Mechanisms of PGPR-induced resistance against pathogens. *In* Plant growth-promoting rhizobacteria. Present, status and future prospects. *Edited by* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino. Nakanishi Printing, Sapporo, Japan. pp. 50-57.

Van Onckelen, H.A., Caubergs, R., and Degreef, J.A. 1977. Effect of light treatment and endogenous growth hormones on α- and β-amylase activities in cotyledons of *Phaseolus vulgaris* L. Plant & Cell Physiol. **18**: 1029-1040.

Van Overbeek, L.S., and Van Elsas, J.D. 1994. Root exudate-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. Appl. Environ. Microbiol. **61**: 890-898.

Van Staden, J., and Dimalla, G.G. 1976. Cytokinins from soils. Planta **130**: 85-87.

Van Staden, J., and Dimalla, G.G. 1978. Endogenous cytokinins and the breaking of dormancy and apical dominance in potato tubers. J. Exp. Bot. **29**: 1077-1084.

Van Staden, J., and Mooney, P.A 1988. The effect of cytokinin preconditioning on metabolism of adenine derivatives in soybean callus. J. Plant Physiol. **133**: 466-472.

Van Staden, J., Cook, E.L., and Nooden, L.D. 1988. Cytokinins and senescence. *In* Senescence and aging in plants. *Edited by* L.D. Nooden, and A.C. Leopold. Academic Press, San Diego. pp. 281-292.

Venis, M. 1987. Hormone receptor sites and the study of plant development. *In* Hormone action in plant development : a critical appraisal. *Edited by* G. V. Hoad, J. R. Lenton, M. B. Jackson, and R. K. Atkin. Butterworths Co. Ltd., Long Ashton, UK. pp. 53-62.

Volkmar, K.M., and Bremer, E. 1998. Effects of seed inoculation with a strain of *Pseudomonas fluorescens* on root growth and activity of wheat in well-watered and drought-stressed glass-fronted rhizotrons. Can. J. Plant Sci. **78**: 545-551.

Wang, T.L. 1994. Cytokinin mutants. *In* Cytokinins: chemistry, activity and function. *Edited by* D. W. S. Mok and M. C. Mok CRC Press Inc., Boca-Raton. pp. 255-268.

Wang, T.L., Everett, N.P., Gould, A.R., and Street, H.E. 1981. Studies on the control of the cell cycle in cultured plant cells. III. The effects of cytokinin. Protoplasma **106**: 23-35.

Wang, T.L., Wood, E.A., and Brewin, N.J. 1982. Growth regulators, *Rhizobium* and nodulation in peas. Planta **155**: 350-355.

Weiler, E.W. 1980. Radioimmunoassays for trans-zeatin and related cytokinins. Planta **149**: 155-162.

Weiler, E.W. 1984. Immunoassay of plant growth regulators. Ann. Rev. Plant Physiol. **35**: 85-95.

Weiler, E.W., and Ziegler, H. 1981. Determination of phytohormones in phloem exudate from tree species by radioimmunoassay. Planta **152**:168-170.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. **26**: 379-407.

Weller, D.M., Howie, W.J., and Cook, R.J. 1988. Relationship between in vitro inhibition of *Gaeumannomyces graminis* var *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. Phytopathol. **78**: 1094-1100.

Weller, D.M., and Thomashow, L.S. 1993. Use of rhizobacteria for biocontrol. Curr. Opin. Biotech. **4**: 306-311.

Whipps, J.M., and Lynch, J.M. 1983. Substrate flow and utilization in the rhizosphere of cereals. New Phytol. **95**: 605-623.

Wilson, M.M., Gordon, M.E., Letham, D.C., and Parker, C.W. 1974. Regulators of cell division in plant tissues. XIX. The metabolism of 6-benzylaminopurine in radish cotyledons and seedling. J. Exp. Bot. **25**: 725-732.

Wyndaele, R., Christiansen, J., Horseele, R., Rudelsheim, P. and Van Onckelen, H. 1988. Functional correlation between endogenous phytohormone levels and hormone autotrophy of transformed and habituated soybean cell lines. Plant Cell Physiol. **29**: 1095-1101.

Young, E. 1989. Cytokinin and soluble carbohydrate concentrations in xylem sap of apple during dormancy and budbreak. J. Am. Soc. Hort. Sci. **114**: 297-303.

Young, S., Pharis, R.P., Reid, D., Reddy, M.S., Lifshitz, R., and Brown, G. 1990. PGPR: is there a relationship between plant regulators and the stimulation of plant growth or biological activity. *In* Plant growth-promoting rhizobacteria: progress and prospects. *Edited by* C. Keel, B. Koller, and G. Defago. International Union of Biological Sciences, Switzerland. pp. 182-186.

Zablotowicz, R., Press, C.M., N., L., Brown, G., and Kloepper, J.W.E. 1992. Compatibility of plant growth promoting rhizobacterial strains with agrichemicals applied to seed. Can. J. Microbiol. **38**: 45-50.

Zhang, J., and Davies, W.J. 1989. Sequential response of whole plant water relations to prolonged soil drying and the involvement of xylem sap ABA in the regulation of stomatal behavior of sunflower plants. New Phytol. **113**: 167-174.

Zhang, J., and Davies, W.J. 1991. Antitranspirant activity in xylem sap of maize plants. J. Exp. Bot. **42**: 317-321.

Zhang, F., Dashti, N., Hynes, R.K., and Smith, D. 1997. Plant growth-promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] growth and physiology at suboptimal root zone temperatures. Ann. Bot. **79**: 243-249.

Zimmer, W., and Bothe, M. 1988. The phytohormonal interaction between *Azospirillum* and wheat. Plant Soil **110**: 239-247.

APPENDIX A

Minimal Medium (MM+Gl)

Component 1:
1.36 g KH_2PO_4
1.74 g K_2HPO_4
in 408 mL of deionized water

Component 2:
0.5 g $MgSO_4$ (anhydrous)
1.0 g NH_4Cl
in 572 mL of deionized water

Autoclave the two components separately to avoid the formation of a phosphate precipitate, mixed after autoclaving and 40 mL of 25% of filter-sterilized glucose (Gl) solution added.

APPENDIX B.

Effect of Gibberellic Acid (GA_3) on Hypocotyl Elongation of Lettuce Seedlings.

GA_3 standard solution treatment [1] ($\mu g\ L^{-1}$)	Mean Hypocotyl Length [2] ± SE (cm $plant^{-1}$)
0	1.03 ± 0.01 e[3]
10	1.25 ± 0.02 de
10^2	1.41 ± 0.02 cd
10^3	1.88 ± 0.03 bc
10^4	2.43 ± 0.03 ab
10^5	2.74 ± 0.02 a

[1] Standard solutions of GA_3 used to obtain dose-response curve (4 ml $plate^{-1}$).

[2] All the bioassays were performed as described by Frankland and Wareing (1960). A linear relationship was obtained between the logarithm of GA_3 concentration and hypocotyl length of seedlings of lettuce variety Grand Rapid.

Hypocotyl Length (cm pl^{-1})=0.999+0.381 (log $\mu g\ GA_3\ L^{-1}$); r^2= 0.99; n=360.

[3] Means followed by the same letters were not statistically different as determined with Tukey's test at the rejection level $P = 0.05$. SE: standard errors of the means.